ANNUAL REVIEW OF BIOCHEMISTRY

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PREFACE

Having been advised that the Board of Directors of Annual Reviews is engaged in a study of several proposals for the organization of Reviews in certain other sciences, we consider that it may be timely to appraise the present status of the *Annual Review of Biochemistry*. How does this *Review* fit into the scheme of things? Does it still perform a useful function? Is it able to keep abreast of the ever rising flood of primary publications in Biochemistry? Are the individual reviews sufficiently critical? Do the authors succeed in giving a fair appraisal of the present status of the subject within the relatively few pages that each has at his disposal? Is there too much duplication of content from chapter to chapter or between neighboring *Reviews* or are there serious gaps—repeated omission of lively areas of biochemical research?

We shall not attempt to answer these and many related questions. We present them to our readers only because we would welcome their comments and advice. Indeed, we suspect that the Board would be glad to learn whether teachers and investigators in the various sciences may not even be frustrated by current increases of considerable magnitude in reviews of all sorts throughout many of the sciences. Perhaps we can sympathize with a distinguished colleague abroad who displayed something bordering on irritation when learning of still another Review. "Too much to read already; what we really need is more time in the laboratory and less time in the library."

The authors of the reviews in this present volume were selected by the Committee about two years ago. Although each had complete freedom of movement within his allotted space, the restrictions that had to be imposed upon length of manuscript and time of preparaton may have seriously curtailed the liberty of those who were eager to make broad excursions and detailed surveys throughout the expanding domains of subject matter that were theirs. We convey to them, one and all, our very sincere thanks for their devoted efforts in a very difficult task. We greatly regret that circumstances entirely beyond their control denied to two of the prospective authors the possibility of completing their manuscripts on time; we refer to the proposed reviews on "Biological Oxidations" and "Cellular Permeability to Organic Metabolites."

Margaret Janofsky has served most helpfully as the editorial assistant principally responsible for seeing the manuscripts through the press. Dr. Gordon Nordby gave us his generous assistance in preparing the subject index. To them we are greatly indebted for their devoted help. Finally we wish to express our thanks to our printers, the George Banta Company, with whom we have continued to enjoy a most cordial relationship and complete co-operation.

H.E.C.	J.M.L.
F.S.D.	E.L.S.
B.L.H.	E.S.

ERRATA

Volume 26 (1957):

    page 619, line 1: *for* Kainova & Petrova (43) *read* Stepanenko, Kainova & Petrova (43)

    page 619, line 7: *for* Khlurova *read* Khaurova

    page 640, line 1: *for* 43. Kainova, A. S., and Petrova, A. N. *read* 43. Stepanenko, B. N., Kainova, A. S., and Petrova, A. N.

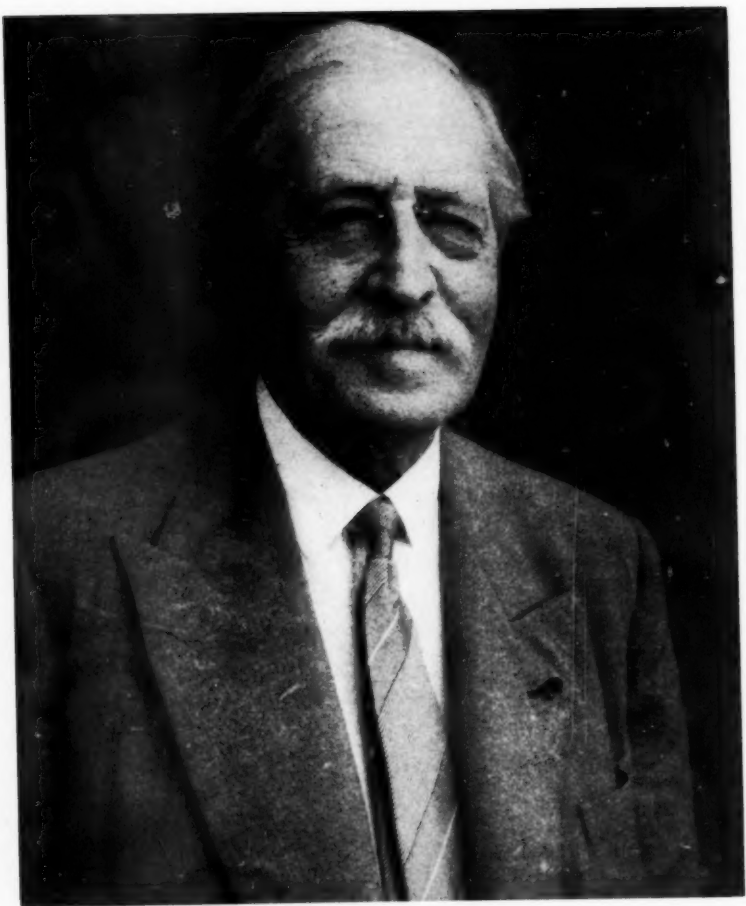   page 640, line 3: *for* Khlurova *read* Khaurova

Volume 27 (1958):

    page 21, structure VI: The AcO- groups at carbon atoms 3 and 4 should occupy axial rather than equatorial positions

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E. T. Peaseisse

PREFATORY CHAPTER

FIFTY-FIVE YEARS OF UNION BETWEEN BIOCHEMISTRY AND PHYSIOLOGY

By Emile F. Terroine

Centre national de Coordination des Études et Recherches sur la Nutrition et l'Alimentation, Paris, France

My first publication appeared on March 21, 1904, in the form of a note to the Academy of Sciences. This was my earliest venture, an intellectual and technical apprenticeship begun the preceding year. Thus the title of the present paper is justified in so far as the period of time is concerned. It is justified also in the attitude it expresses, for one idea has dominated my whole scientific career from the very beginning up to the present time of writing—the belief that the indissolubility of the union between biochemistry and physiology is an essential requirement for the progress of both. Without biochemistry, physiology is unable to undertake a close analysis of the processes it observes and records; biochemistry, outside its physiological context, is in danger of losing touch with reality and of confusing potentialities with facts as they are. I shall take two examples from the first phase of my career to illustrate this attitude and the results that may be expected from it.

At the time I have in mind, studies *in vitro* had shown that the pancreatic juice acts on starch to convert it into maltose. It was concluded from this that the successive action of the pancreatic juice and the intestinal juice was required to effect the complete transformation of starch into glucose. With Bierry, we recalled that, under physiological conditions, the pancreatic juice does not act in its own alkaline environment, but, rather, in the neutral or slightly acid environment which results from the mixing in the intestine of acid chyme, pancreatic juice, and bile. We were then able to show that after mere neutralization of the juice, the attack very quickly produced glucose, thus revealing the presence of very active maltase in the pancreatic juice.

Then again, there was at that time a generally held belief that proteolytic activity was completely lacking from pure pancreatic juice, drawn off with great care and uncontaminated by the intestinal juice. This belief was based on the correctly observed fact that this juice has no effect on native proteins, but it ignored the fact that in the intestine the juice's main action is not on native proteins, but on their degradation products, formed by the action of the gastric juice. By causing the pure juice to act on these products—acid albumins, proteoses, peptones—Schaeffer and I showed the rapidity of its attack. This brought to light the presence of an enzyme in the juice, which at that time we incorrectly called an erepsin, according to the terminology of

the day, but which was actually carboxypolypeptidase. Thus the juice was able to effect the complete transformation of proteins into absorbable products; this discovery subsequently explained why there is no change in the rate of digestive utilization of proteins either after total gastrectomy or after the removal of four fifths of the intestine. At a later date, I was to show, first with Przylecki and then with Mme. Kahn-Marino, by use of procedures in which biochemical and physiological techniques were closely interlinked, that the essential role of the pancreatic juice in the digestion of proteins is played by peptidase, while trypsin plays only a secondary part in acting on the residue of native proteins that have escaped the action of the gastric juice.

Now, toward the close of my career, I remain more than ever convinced of the need for this close union between biochemistry and physiology. Even if the increase in the number of techniques and in their diversity makes specialization inevitable, it must not lead to a divorce between physiologists and biochemists, for this would render the former unproductive and threaten to make the latter forget the complexity of vital phenomena.

I should perhaps describe at this point how I came to have the desire to apply myself to the study of the biochemical aspects of physiology.

Born in Paris in 1882 of parents of very modest means, I received my primary education at the local elementary school in a very humble neighborhood. I shall never forget the quality of the teaching at that school. If I was able to start my secondary education with a solid basis of elementary knowledge, and if I acquired the habit—an unconscious one at that age—of thinking methodically, of sorting out my ideas and stating them correctly, I owe it to the teachers there, and I am profoundly grateful to them.

The Chaptal Lycée—or Municipal Secondary School as it was at that time—to which I went at the age of 13, was a somewhat exceptional institution for those days. A good many of the teachers were very young and had no intention of making a permanent career for themselves in secondary education; they gave all their free time to research and were soon to pass on to the field of higher education. This was true of the chemists Maquenne and Rivals, the psychologist Georges Dumas, and the sociologist Edgard Milhaud, among others. Thus their teaching was full of life and permeated with new ideas; they made every effort to foster a liking for individual study. It was under this influence that, in spite of the advice of some people who wanted me to take up a literary career, I felt myself drawn at an early age to the experimental sciences. I can still see myself and my friend Georges Schaeffer using our very slender resources to buy chemicals and glassware, spending our free days in trying to make the chemical preparations about which we had learned at school and, naturally, being attracted by the most dangerous ones.

Entrance to the University—to the Science Faculty in Paris—was a severe test. There was in those days no department responsible for giving students information or advising them on the subjects to which their abilities and preferences were best suited. I spent some time mistakenly studying the

morphological sciences, the value of which I would certainly not deny, but for which I was simply not fitted. A chance meeting brought me into contact with one of the most original and fascinating scientific personalities I have known, Victor Henri. Thanks to him, I was able to make a better choice of subjects for my degree in science, and, even before I had graduated, he introduced me into the Physiology Laboratory at the Sorbonne. He had set my foot on the road which I was never to leave.

However improbable the assertion may seem to some people, particularly the younger generation, I have no hesitation in saying that there was at the time I was beginning my career a truer and deeper respect for science than there is today. Science was an ideal in itself, not a mere tool for technical progress. In the wake of the great thinkers of the nineteenth century—such as Auguste Comte, Spencer, and Renan—what people admired most in science was its function in freeing the human mind and its triumphal progress toward the extension of positive knowledge. And even when they contemplated the practical application of its conquests, people cherished the illusion that increase in the well-being of mankind would soon lead to the establishment of lasting peace.

Although this conception was supposed to be positive and rational, it retained a certain religious element. The scientist, still somewhat aloof from his time, had a sort of priestlike function for which a special calling was required; this function could not be taught. Science was not, as it is for many today, an occupation like any other, whose material advantages and disadvantages a young man weighs before engaging in it. None of those organizations existed which we now see springing up everywhere for the training of research workers. There was nothing for young men such as I or my fellow students, whatever the branch of science they chose, but a sort of early apprenticeship in the laboratory, where they learned by working with their seniors, with some general advice from the Director now and then. My Director, Dastre, a man of the most eminent intellect who had been a pupil of Claude Bernard himself, said to us one day, toward the end of his life, "The thing that I am proudest of is that I brought you up in freedom." His method implicitly demanded considerable personal effort from the young research worker, who could be successful only if he possessed the essential qualities of the man of science—imagination, tenacity, and a passion for research.

I am not convinced that all the institutions being developed today to provide training and assistance for research workers by means of longer and more intensive instruction, and of adding to their store of knowledge without requiring of them any great personal effort, will produce better results than our education in freedom.

It was in this atmosphere of liberty, and of comradeship with those whose names were later to make their mark in biological physics, biochemistry, and physiology—particularly Victor Henri, André Mayer, Georges Schaeffer, Leo Ambard, and Jean Giaja—that the first phase of my scientific career was to develop.

At that time, the Physiology Laboratory of the Sorbonne was, so to speak, drawn up in battle formation. Because our investigative procedures and our state of mind clearly indicated a physicochemical tendency, many people wanted to deny us access to physiology. But we were certain that we were on the right road. After all, we had followed in the footsteps of Claude Bernard, who was still very close to us, in the laboratory which he had founded and where Dastre, who continued his work, passed his ideas on to us. Did we not have splendid proof of the necessary interdependence of biochemistry and physiology in the discovery of glycogen and of the very essence of the mechanism of blood-sugar regulation, that of the mechanism of digestion and of fat absorption? Furthermore, had not Claude Bernard written in 1867, in his masterly *Rapport sur l'état de la Physiologie en France,* "Physiology, i.e. animal physical chemistry and plant physical chemistry . . ."? It is therefore not surprising that our group actively participated in the creation of the *Société de Chimie Physique* in 1907 and played a leading part in setting up the *Société de Chimie Biologique* in 1913.

The First World War was to interrupt this eager activity for a long time. When the provinces torn from France in 1871 were restored to her and the University of Strasbourg was reconstituted, I had the great honor of being appointed to the Chair of General Physiology at the University's Faculty of Science and of setting up a completely new Institute of Physiology. I was, no doubt, guided by my own personal inclinations in undertaking this task but, here again, my inclinations were in keeping with one of the governing ideas of the scientist and philosopher whom I worshipped. In the report I have just quoted, Claude Bernard had also written, "The real viewpoint of physiology is, as it were, the *nutritionistic* one." The eminent physiologist meant by this the ensemble of all the processes which constitute the basis of all living matter, the degradation and simultaneous renewal of all its constituents. It was thus to expand our knowledge both of the general processes of nutrition and of its inmost mechanism—later to become the science of intermediary metabolism—that this magnificent Institute was built and equipped. During the 20 years which I spent in Strasbourg, the Institute admitted 108 research workers, 86 of them French and 22 foreign; their activity was recorded in the publication of 203 memoirs, in addition to short notes.

But alas, when this Institute was in the course of rapid development, I was doomed once again to give up all productive activity in the field of research. The war, followed by enemy occupation, and the nonscientific tasks which I was in duty bound to accept afterward took me almost entirely away from scientific activities for nearly seven years, despite my efforts not to lose touch completely with foreign publications.

When the younger generation of scientists, who are always very critical, weigh the contribution made by the men of science of my generation and in many cases find it wanting, they should not forget that these men sacrificed at least ten years—often more—of their scientific life, perhaps the years

which would have been the most fruitful. Those of the younger generation should also be mindful of the rude awakening which may well awake them, in the increasingly troubled times in which we live, if they content themselves with pursuing their research with the often blind passion of the man of science, in the confined atmosphere of the laboratory, and if they do not strive to turn the formidable power which they hold in their hands into an instrument of peace.

The Liberation, while marking the triumph of the human values to which all civilized men are so deeply attached, was not without its sadness at the magnitude of the losses suffered. There was, to be sure, no justification for complaint where purely material losses were concerned. None the less, I was filled with resentment when I learned that the Institute of General Physiology in Strasbourg, to the foundation and operation of which I had given of my best, and which had been the finest French laboratory of its time for biochemical and physiological research in nutrition had been totally destroyed by the occupying power in a wanton act for which the motives remain incomprehensible.

When I was free once again to devote myself to science, I was nearly 65 years old and thus only a few years short of retirement from university teaching. At that time, any restoration work encountered considerable practical difficulties. Shall I admit it?—I had not the courage to start all over again, to rebuild the institution that had been destroyed or to undertake a task that I should not have had time to see through to completion. Furthermore, since a completely fresh start was needed, I thought it preferable to leave it to a younger man.

I considered that the last years of my life could make a more useful contribution to scientific progress if I played the part of adviser, which is the prerogative of old men, and of organizer. It was at this time that the *Centre National de Coordination des Études et Recherches sur la Nutrition et l'Alimentation* was created and charged with the task of co-ordinating all nutritional studies for the whole of France and establishing the necessary contact between the pure scientists concerned with these studies, on the one hand, and the food producers and food industry experts, on the other. I was appointed its first Director, in 1946, and I hope I may continue to discharge these functions as long as some activity is left to me.

Engaging in and directing research activities; teaching; writing books which present a synthesis of my studies; and, at the same time, making use of the competence acquired in this way by taking part in activities of general interest—these are the four aspects of my scientific life.

RESEARCH

When I entered the field of research, I felt the necessity of broadening my physiochemical knowledge, and likewise felt a desire to engage in productive activity. During this period, my work lay essentially in the physiochemical sphere, judiciously guided by my seniors, Victor Henri and André

Mayer, whose co-worker I became. In collaboration with the former, I formulated in 1904 what we called "Duclaux's Law," which expresses the influence of the concentration of the substrate on the rate of action of an enzyme. With the latter, between 1905 and 1907, sometimes in association with Schaeffer, I undertook a whole series of studies on the colloidal characteristics of the various constituents of living matter. This investigation led us accurately to define the role of the reaction of the medium in determining the extent to which particles combine with water; to obtain alcoholic protein solutions through the presence of certain electrolytes; to show, in the series of sodium salts of saturated fatty acids, the existence of a relationship between the size of the molecule and the appearance of colloidal phenomena; to define the properties of lipoprotein compounds and to produce certain synthetic ones.

While continuing to collaborate with André Mayer in certain areas, I soon embarked on more personal activities. Apart from a few incidental, temporary interests (existence of a general constitutional law for all warm-blooded animals; cultivation of the tubercle bacillus in a chemically defined medium; definition of the nitrogen requirements of this microorganism; constancy of composition of the egg; composition of microorganisms, with particular reference to nucleic acid content, etc.), these activities fall, no doubt with some overlapping, into three periods devoted respectively to fat metabolism, bio-energetics, and nitrogen metabolism. It is a very hard task, colored by pride and subject to serious errors, to try to single out for myself, among all these activities, those which seem of some importance. But since, after some hesitation in the face of the difficulty of my task, I accepted the hazardous honor done me by the Editorial Committee of this *Review,* I have no alternative but to attempt this intellectual self-examination, as it were, as objectively as I can.

Fat metabolism.—The dominant fact is the establishment of a distinction in every organism between two categories of lipides which are fundamentally different both in chemical constitution and in physiological properties —the constant element and the variable element. Next in order are:

(*a*) The distinction, in the constant element consisting of the complex lipides, especially phosphatides, between "functional phosphatides" and "structural phosphatides;"

(*b*) The determination of the limits of quantitative variation of the variable element in the different animal subkingdoms and the determination of the variables of all kinds (food, temperature, etc.) which govern these qualitative variations in all living beings, particularly the influence of temperature on the proportional relationship between saturated and unsaturated fatty acids;

(*c*) The determination of the essential function of the variable element (triglycerides), which is to provide, almost on its own, for the organism's energy requirements in the absence of a supply of food and to constitute practically the only means of defending the organism, as far as energy re-

quirements are concerned, against the precariousness of the food supply;

(d) A detailed analysis of the conditions under which pancreatic lipase acts, which made it possible to establish the importance of the reaction of the environment, the significance of the intervention of reaction products, the role of activators and, in particular, the fact that activation by the bile is to be ascribed to the bile salts;

(e) The study, also in detail, of the variations in intensity of lipase attack according to the structure of the substrate, which brought to light, among other facts, the almost total resistance of the esters of isoacids and divalent acids, the retarding influence of the presence of hydroxyl groups in the acids, and, above all, the increasing resistance of the glycerides of higher fatty acids in going from tri- to mono-, the latter being hardly affected at all.

Bio-energetics.—In 1922 a work on the energetics of growth in *Sterigmatocystis nigra* marked my first approach to bio-energetics, which had always keenly interested me, but from which I had held back because I feared that my knowledge of physics was inadequate. It was thanks to R. Wurmser, my assistant at the time, that I ventured into this field for, in the study in question, our ideas were closely interlinked; it is my pleasant duty to record that beyond any doubt he played the major part. In essence, our work formulated for the first time all the various conditions which are necessary to draw up an accurate energy balance during growth and to calculate actual energy output; it showed that the energy loss, in the form of heat recorded during the process of growth, corresponds almost exclusively to the chemical operations ending in synthesis, while so-called structural expenditure accounts for very little; and it outlined the fundamental distinction between independent oxidation and coupled oxidation, to which Wurmser was to refer so frequently and judiciously.

Basing my work on the data obtained in this way, I was able later, by a large number of investigations concerned with the development of microorganisms and with the growth of higher plants during germination, to measure the over-all energy output of the main biological processes (formation of carbohydrates at the expense of lipides and proteins; formation of lipides at the expense of carbohydrates and proteins) and to forecast accurately the amount of energy output in growth (germination, for instance) on the sole basis of knowledge of the materials used.

In the particular field of the homeotherms, I contributed, with Lapicque, to a demonstration of the fact that the surface area law is limited to the heat production of basal metabolism. I succeeded in showing that it was impossible to explain the differences in heat production between warm-blooded animals on a weight basis, in terms of differences either in the size of their active mass or in cell composition, and I reached the general conclusion that the essential factors which govern the intensity of combustion do not lie within the cell itself, but are carried to it by the blood stream.

Nitrogen metabolism.—In 1913 I began to take an interest in the prob-

lems which were to hold my attention longest and to give rise to the largest number of investigations and the most extensive ones. These were the problems relating to nitrogen metabolism. By studying them, I was able, among other things:

(*a*) To establish a fundamental distinction in metabolism as a whole between contingent expenditure and necessary expenditure, functional requirements and structural requirements, and differentiated requirement and undifferentiated requirement, specifying the physiological significance of each of these and, for some of them, determining their size;

(*b*) To formulate the law which, in all warm-blooded animals, governs the amount of nitrogen expenditure required, and to show that this expenditure is dependent upon energy expenditure;

(*c*) To determine the distinctive characteristics of contingent expenditure (total inanition, nitrogenous alimentation) as against necessary expenditure; the conditions governing the magnitude of the latter; the characteristic differences between species and individuals in the intensity of the various processes (usage of protein and of nucleic acid, waste production of creatinine) of which the necessary expenditure is the sum total;

(*d*) To define the means of determining the characteristic coefficients of the protein value of a food;

(*e*) To demonstrate, by a variety of procedures, the utilization of ammonium salts as a source of nitrogen in monogastrics, both in maintenance and in growth;

(*f*) To show that it is possible considerably to reduce the waste production of urea to the point of making it disappear almost entirely and be replaced by a waste production of glycine and ammonia, without any ill effects on the subject;

(*g*) To demonstrate conclusively the extensive synthetic formation of purines in adult mammals at the expense of proteins;

(*h*) To give a comprehensive picture of the successive steps in the catabolism of the amino acids introduced into the organism by food proteins;

(*i*) To discover the creatinuria of thermogenesis;

(*j*) To specify the factors governing the amount of creatinuria.

TEACHING

When, in 1919, I was appointed to the Chair of General Physiology of the Faculty of Science at Strasbourg, no provision existed for the teaching of biological chemistry. I set out forthwith to remedy this deficiency and, as part of the Degree course in General Physiology, I devoted a number of lectures to the chemistry of the constituents of living matter, the mechanism of enzyme action, oxidation reduction, and the relationship between the constitution of substances and their biological properties. In 1930, thanks to the co-operation of one of my first students, R. Bonnet, I had the great pleasure of officially introducing a new subject into the Science Degree course at Strasbourg—the subject of biological chemistry, which was to

prove very attractive to the research chemists, doctors, and pharmacists receiving their training at our university.

I have rarely known any greater satisfaction than that I derived from my teaching—not only through the close contact with young people and the feeling of doing something directly useful, but also because of the valuable guidance teaching offers to one's own research activities. Honest teaching often forces us to see a problem in a truer perspective than before. I do not know whether the story is true that Nernst declared that he had never really grasped the implications of Carnot's principle until the day when he had to teach it. Certainly I had a similar experience with some important aspects of biochemistry and physiology.

If teaching is a school of modesty in that it often makes the teacher aware that he has made only a small contribution to the body of knowledge that he is imparting, it is at the same time one of the processes which yields the greatest number of guiding ideas for research. We often find that a discovery, however small it may be, is a revelation of the links between categories of phenomena which had previously appeared unrelated. Teaching imposes comparisons which a research worker who concentrates on one limited type of investigation is not always inclined to make. To take an example—would I ever have thought of formulating the law which provides a quantitative link between the minimum endogenous nitrogen expenditure and basal energy expenditure if I had not come to teach both nitrogen metabolism and bio-energetics? Therefore, while I realize that in applied science research and teaching can be partially dissociated because, in this field, the research worker is always in contact with reality and the problems force themselves on his attention, I believe in the need for a combination of teaching and research in the basic sciences. I am very much afraid that, except for those who are unusually imaginative, scientists who today devote themselves exclusively to research, with insufficient access to the outside world, will not know after a time to what end to direct their research.

I would add that I attribute a great deal of moral satisfaction to teaching. Is there any man of science who, when called upon, as I am today in writing these words, to undertake at the end of his career what I have earlier called an "intellectual self-examination," would have the courage to declare himself satisfied with his achievements and would not be filled with sadness at the realization of how limited in quantity and quality his contribution has been? Is there anyone who could close his eyes, lulled by the serenity of dreams fulfilled? After all, Claude Bernard himself presents the distressing spectacle of this sense of nonfulfillment in expressing regret, shortly before his death, at having been unable to prove—this was to be done twenty years later—that alcoholic fermentation does not require a living organism, but can be effected by an enzyme. But a man may depart in peace if he can have the conviction that, apart from his modest personal achievement, he has through his teaching given new generations the means to add new bricks to the collectively built, constantly developing, edifice of

scientific knowledge. To place myself in this position, I endeavored to imbue my teaching with the spirit which I believe to be appropriate for university teaching in the basic sciences. The Masters should, no doubt, give their students new knowledge, but this is not where their essential duty lies, for, in doing so, they may only be providing teaching of the primary- or secondary-school type with a mere broadening and deepening of content. Their main duty lies in training the students' minds, in stimulating them to acquire new knowledge on their own, in showing them that most new knowledge raises more problems than it solves, and in making them dissatisfied with human ignorance. If their curiosity be aroused, they become anxious to add new links to the continuous chain of scientific progress and not to be content to play the part of mere users by contributing the dross all too common in our modern production.

French Faculties of Science responsible for the teaching of the basic sciences made it possible, when I entered the University, to foster this state of mind, and they still do so, by virtue of their structure and their methods of teaching. There is a limited amount of formal instruction for each main branch of knowledge—but such tuition goes right to the root of the matter—and long vacations leave the students ample time to make a personal effort to enrich their store of knowledge and to train their minds by reflection.

Should I add that I feel uncomfortable in the midst of the general tendency to increase the amount of formal tuition everywhere and to guide the students' intellectual life more and more closely; to leave them less and less free time; and to replace personal effort by guided activity? This tendency is perhaps a very good thing from the point of view of the teamwork which is so highly extolled today, and which, I admit, has its value in certain sectors, but I do not believe that it is a good way to encourage development of the type of individual which basic science needs more than anything else.

BOOKS AND REVIEWS

As far back as I can remember, I have always had a liking for comprehensive reports and a desire to find a place for my personal studies in a broader setting than the necessarily limited one of notes and memoirs. Here again, I may well have been under the influence of Claude Bernard, in whose wonderful succession of works I had taken a passionate interest at an early age. May I be permitted to state—and this will be readily understood after what I have just said—that I have no liking for the vast majority of present-day publications, which consist of a more or less coherent collection of articles by a wide variety of authors and whose primary purpose is informative, not synthetic and interpretive. These are works which are mostly consulted, not read. For me, a book is something strictly personal and original. It is a structure, as complete as it can be made with the materials available. The author is not a mere onlooker; he takes sides in controversies; he does not hesitate to make known his own way of looking at

things; he tries to show the way which he believes should be taken to achieve progress in the study of the problems with which he is dealing. He knows that his work cannot be permanent, that it may even be short-lived in view of the present rate of scientific production. But he also knows that, in pointing the way to others, his work will serve as a milestone; even if it is short-lived, it will not have been in vain. Then there is the great satisfaction of knowing that it may help those who later follow the same road.

It was in this spirit that, in 1913, I wrote my first book, *La Sécrétion Pancreatique,* at a time when physiologists were beginning to take a keen interest in the new regulatory agents, hormones, the vital importance of which had been brought to their notice by the magnificent discovery of secretin in 1902 by Bayliss and Starling.

In 1925, the publication of *Métabolisme de base* marked the period during which my main activity was directed toward bio-energetics.

At this time, when I was committed to the road which I was to follow in the future, I formed the plan of presenting all the aspects of nitrogen metabolism in comprehensive form. A first volume, *Dépenses, besoins, couverture,* which set forth the general problems and the broad laws of nitrogen metabolism, appeared in 1933. This was followed in 1936 by a second volume, *Physiologie des Substances protéiques—aliments, digestion, absorption.* In 1939, 1940, and 1941 three sections on protein catabolism appeared; these dealt with (*a*) Characteristics, conditions, general mechanisms, oxidizing and regulating agents; (*b*) The common characteristics of amino acid catabolism, ureogenesis and uricogenesis, formation of ternary substances, combustion; and (*c*) The evolution of individual amino acids. The three last were little known in view of the date when they were published. When I was able to return to work after the long crisis of the Second World War, I continued with the preparation of a book on which I had started earlier, *La Synthèse protéique,* which finally appeared in 1952. The long interruption resulting from this period of crisis will certainly prevent me from completing the overambitious project I had undertaken. I hope, however, that I shall be able fairly soon to complete the manuscript of *Le Métabolism des Substances nucléiques.*

PARTICIPATION IN ACTIVITIES OF GENERAL INTEREST

On many occasions I have expressed my conviction that thought in all its forms is the mainspring of all human progress; thus I have never ceased to believe in the pre-eminence of pure science. At the same time, I do not think that a scholar is being disloyal when he devotes part of his time to action, by collaborating in work of general interest, if his temperament drives him to do so and he is aware that he has the ability to make a useful contribution. Such were the activities in which I was engaged, almost involuntarily at first; during these last years they have come to take up the major part of my time. They became part of my life during the First World War, when, in face of the use of gas by the enemy on April 22, 1915, it was

necessary to find some means of protecting our troops against such attacks
and of countering them. I gave up all my time to work for the joint scien-
tific and military bodies which made these means available; I held the post
of General Secretary of the Army Chemical Research Department and was
soon also entrusted with the tasks of organizing an interallied secretariat
and of serving as technical adviser to the American Army for the estab-
lishment of its research organization in France. It is true that it cost my
colleagues and me a painful conflict of conscience to resign ourselves to such
activity, imbued as we were with the idea that science should serve only to
improve man's lot. But had we the right, in not giving our assistance, to
risk placing our armies in a position of manifest inferiority, the certain re-
sult of which would have been a servitude intolerable to any man for whom
freedom is the most precious possession? Our foreign colleagues had to
face this conflict of conscience as we did, and in particular I well remember
long conversations on the subject with one of my seniors, the eminent Eng-
lish biochemist and physiologist of great moral integrity, Joseph Barcroft.

After the war, I remained attached to the Army Chemical Research De-
partment until 1940, with the task of training young people to play an active
part in this field. During the occupation of France in the last war, I en-
deavored to maintain and develop in the young people around me a state
of mind and attitude uncompromisingly hostile to Hitlerite ideas and to the
activity of the Vichy government; at the same time, I tried to help those
who were in the greatest danger under the occupation, especially Jews. Such
behavior resulted in my arrest and confinement in the notorious prison of
Montluc at Lyons, where I had the unexpected good fortune to escape the
firing squad, or deportation at the very least, thanks to the arrival of the
American and French forces. I recorded my experiences and those of my
fellow prisoners in a little volume which appeared at the end of 1944, *Dans
les Geoles de la Gestapo. Souvenir de la Prison de Montluc.* Immediately
after the Liberation, I was placed in charge, in the Rhone-Alps region, of a
department for the restitution, to victims of the confiscation measures taken
by Germany and the Vichy government, of the possessions of which they
had been robbed. In January 1945, I was appointed director of a similar
department for the whole of France and entrusted with the task of draw-
ing up the legal provisions required for such restitution and to supervise
their application. In doing so, I made every effort to play my part in un-
doing—only too inadequately—the consequences of the shameful measures
of racial discrimination and the robbery they involved. The most frequent
victims had been our Jewish compatriots.

During this time, from September 1944 to May 1946, I gradually re-
newed contact with science. It was then, in the circumstances which I found
at the time and which I have described earlier, that I planned the creation
of an institution designed to co-ordinate and foster the efforts of all those
in France concerned with nutritional problems. *Le Centre National de Co-
ordination des Études et Recherches sur la Nutrition et l'Alimentation* was

created and placed under my direction. Development of pure scientific research (in biochemistry, physiology, bacteriology, pathology) connected with nutrition; establishment and maintenance of very close relations between scientists and technicians (agronomists and food manufacturers); creation of permanent working groups with a view to increasing the production of foodstuffs and improving their quality; teaching of the newest techniques in order to give advanced knowledge to scientists and young technicians; dissemination to all those interested in the broad problems of nutrition and food, with up-to-date information on the state of progress of the varied theoretical and practical aspects of these problems—such were, and still are, the activities of this new institution. In the sphere of publications, they manifested themselves in a new periodical, *Les Annales de la Nutrition et de l'Alimentation,* now in its twelfth year, and in various books, each of which deals with one basic food: *Le Pain* (1948), *Les Corps Gras Alimentaires* (1949), *La Production du Lait* (1950), *La Production de la Viande* (1951), *La Volaille et l'Oeuf* (1953), *Le Lait stérilisé* (1954), *Les Fruits et Légumes* (1955).

Finally, I should like to mention my participation in the activities of the specialized agencies of the United Nations. The ground for this was prepared by my faith in international organizations. When a Standing Committee of Experts on Nutrition was formed to assist the Nutrition Division of the Food and Agriculture Organization, I was invited to become a member, and I continued regularly to collaborate with it when it became a joint Food and Agriculture–World Health Organization Committee. In addition, I had the honor of chairing a Committee established by these organizations with the object of formulating a set of recommendations on the protein requirements of the human being and the means of satisfying them.

Absorbing as my duties in research, teaching, writing books, and taking part in collective activities were, I always made sure that, apart from particularly busy periods, they left me sufficient time for totally different occupations. Hardly a day has gone by without my spending at least two hours reading political, historical, artistic, and especially literary works from other countries. Life would have seemed incomplete and perhaps rather colorless, if it had been limited to only my professional activity and if I had not felt a closer bond with my fellow men through knowledge of political life, in which I played an active part in my youth and, even more, through a liking for literary and artistic works which has constantly developed. No doubt these interests stemmed in part from a natural inclination; the latter was, however, fostered and developed to a considerable extent by the French secondary education to which I owe so much because it maintains a harmonious balance between literary and scientific subjects; because it rightly considers that writing an essay and solving an algebra problem are of equal value in training the mind; because it does not permit practical work in experimental science to exclude visits to museums; because, in short, it seeks to produce not specialists, but men to whom no

human activity is foreign. There is the origin of my unshakeable hostility to all the attempts being made more or less everywhere to lay much greater emphasis in secondary education on science teaching, at the expense of literature, history, and the arts. This may perhaps serve to provide more quickly and in greater numbers the more and more highly specialized technicians whose training appears to be the main concern of the modern world. But I do not think that the training of men of science will be improved thereby or—and this is far more important in my eyes—that the human value of each individual will be developed in this way.

I should like to conclude by recalling, now that I have reached the end of my career, what I wrote *in fine* in the preface to my doctorate of science thesis 40 years ago: "In presenting this study today, I cannot fail to remember or to state that if I was able to undertake it and pursue it, I owe this to democracy. Having been a pupil of the local elementary school, the holder of a scholarship at the Chaptal Municipal Secondary School, of a free place at the University and of a doctorate fellowship at the Natural History Museum, it is because I benefited by these advantages . . . that I can today solicit the highest degree accorded by the University to one of its workers. . . . I know what I owe to French democracy; my ambition will never have a nobler objective than to be its most devoted servant."

I hope that I have not failed to keep this promise.

E. F. TERROINE

CHEMISTRY OF CARBOHYDRATES[1,2,3]

By Ward Pigman, K. Nisizawa, and S. Tsuiki

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Scope and Literature

The rapidly expanding literature in the general field of the carbohydrates necessitates limitation of the present discussion to a few topics. Since previous reviews have tended to cover purely chemical aspects or products of plant origin, the topics of the present review will be centered in work of biological interest, especially relating to animal tissues. In the previous review in this series, Reeves applied current concepts of ring conformations to carbohydrate reactions (1).

A number of reviews have appeared of some important topics of carbohydrate chemistry and biochemistry. Of special interest are two comprehensive texts, one by Micheel & Klemer (2) and one edited by Pigman (3), Honoring B. F. Helferich, *Angewandte Chemie* [69, 405–38 (1957)] published a special issue with the following reviews: the glycosidases and special phases of carbohydrate chemistry (H. Brederick), the synthesis of products in intermediate metabolism (H. O. L. Fischer), starch and Schardinger dextrins (K. Freudenberg), oligosaccharides (M. G. Blair and W. Pigman), and polysaccharides of grasses (H. H. Schlubach).

The *Proceedings of the Fourth International Congress for Biochemistry* (Vienna, September 1958), *Symposium I*, provide reviews of seaweed polysaccharides (C. Araki), oligosaccharides (J. E. Courtois), plant gums (E. L. Hirst), sialic acids (G. Blix), immunological behavior of polysaccharides (M. Heidelberger), phenylhydrazones and derivatives (L. Mester), biosynthesis of sugars (J. K. N. Jones), sugars of cardiac glycosides (T. Reichstein), carbohydrates of nucleic acids (M. Stacey), hemicelluloses (R. Whistler and J. L. Sannella).

Proceedings of two conferences on the polysaccharides and glycoproteins of animal tissue and of microorganisms have been published (4, 5). The amino sugars and sialic acids have been reviewed by Kuhn (6), and amino sugars and glycosylamines by Heyns (7) and Baer (7a).

Advances in Carbohydrate Chemistry continued its invaluable series with the appearance of Volume 13 (1958). A special monograph on methyl glucoside appeared (8) and Hirst reviewed the polysaccharides of marine algae in his presidential address to the Chemical Society of London (9).

GLYCOSYLAMINES

Greater appreciation of the biological significance of the formation of glycosylamines and of the possibilities of rearrangements has accelerated studies of these compounds. The glycosylamines form easily from reducing sugars and amines under conditions similar to those in cells, although their formation is best under anhydrous conditions (3). Many dissociate readily in water, but the nature of the groups attached to the nitrogen markedly affects the rate and extent of hydrolysis. Recent studies have confirmed earlier conclusions (10) that even in neutral aqueous solution amino acids combine with D-glucose, although the equilibrium usually favors hydrolysis (11, 12).

Glycosyl derivatives of a number of amino acids (13) are stabilized by the formation of a chelate structure in methanol solution in the presence of the chlorides of some salts ($CaCl_2$, $ZnCl_2$, $FeCl_3$, $CoCl_2$, $CuCl_2$). Some of these products are crystalline. Divalent cations form chelates with two moles of the glycosylamine. The possibility of the stabilization of glycosylamine linkages in biological systems by such chelation should not be ignored.

The weak base urea required acid for its condensation with glucose, galactose, and lactose, but the catalytic activity of several acid types followed no logical sequence (14).

Ammonia reacts with aldoses or their acylated derivatives to give glycosylamines or diglycosylamines, and new compounds of this type have been described from D-xylose, L-arabinose, D-galactose, D-mannose, and sorbose (15, 16). The fully acylated sugars react with ammonia to produce N,N'-acylated aldosylidenediamines [$HC(NHCOR)_2$] (17).

The glycosyl ammonias hydrolyze quickly in aqueous solutions of weak acids (pH 4 to 5) but are only slowly hydrolyzed by stronger acids and by bases. These results may be of considerable interest in biological systems and may explain some phenomena for systems which may involve reactions with nitrogen atoms, such as optimal pH of enzyme action and changes of viscosity or of molecular combination in weakly acidic solutions.

The key steps in the formation of glycosylamines and their hydrolysis probably involve carbonium ions:

$$\text{R—NH}_2 \quad \overset{H}{\underset{|}{^+C}}\text{—OH} \rightleftarrows \text{R—N—}\overset{H}{\underset{|}{C}}\text{—OH} \rightleftarrows \text{R—N—}\overset{H\ H}{\underset{|}{C^+}} \text{ OH}^-$$

$$\qquad\qquad (I) \qquad\qquad (II) \qquad\qquad (III)$$
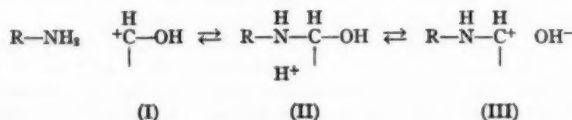
This mechanism is similar to that given by Isbell & Frush (15) and agrees with the observation that the reactions proceed best with strongly basic amines which react directly and displace the oxygen as hydroxyl ions or equivalent. Acids are catalysts since they aid in the formation of the carbonium ions (I, III) formed from the ring structures by addition at the ring oxygen (15) or by addition to the imino nitrogen or carbonyl oxygen of the open chain form. The reactions of weakly basic amines or of ketoses especially require acid catalysts. Weak acids are good catalysts because they provide both acid and base species, and the acids are not strong enough to protonate the reacting amine (synthesis) or the glycosylamine (hydrolysis) completely into nonreactive forms. Although Isbell & Frush found that glycosylamines were stable in hydrochloric acid solutions, the acid was present in excess; with small amounts present as in the formation of ketosylamines (18), hydrolysis occurred.

In agreement with this mechanism, the stability of the N-glycosyl linkage in some acetylated glycosylarylamines decreased with increasing basicity of the amine. Acetylated structures were more stable than the unacetylated glucosylamines (19).

However, adequate studies of the mechanism of such reactions are still lacking, and the basic problem of whether open-chain or ring structures are involved is unsettled. Also, generally the effect of the ratio of acid catalyst to base has not been studied, and the base strengths of the glycosylamines have not been measured.

The α-D-mannopyranosylamine mutarotates more rapidly than those of other aldoses and also shows the anomalies of rotation previously found for mannose and derivatives. Isbell & Frush (15) point out that α-D-mannopyranose in the boat (B1) conformation is probably more stable than in the chair (C1) conformation found for most pyranoses (1).

Further examples of the formation of ketosylamines have been provided by Heyns et al. (16) and by Knotz (18); acid catalysts are required. The greater reactivity of aldoses in this reaction corresponds to the generally greater reactivity of aldehydes in comparison with ketones. With phosphorus oxychloride as catalyst, Knotz obtained Schiff bases of hydroxymethylfurfural (18). This type of product is of special interest since it has been postulated as an intermediate in darkening reactions involving glycosylamines.

An enzyme in Takadiastase transfers a fructofuranosyl group from sucrose to aniline to form N-phenyl-D-fructosylamine (20), identical with that described earlier by Barry & Honeyman as a fructopyranose (21). If the fructosylamine really has a pyranose structure, the reaction may be of considerable interest from the standpoint of the mechanism of the reaction. However, since the structure was determined by hydrolysis of the tetra-acetate, acetyl migrations may have occurred at some point.

A peculiar effect of the presence of water in determining the type of isomer obtained in some preparations of glycosylamines now seems to have

been misinterpreted. The same isomer of N-*p*-tolyl-D-glucosylamine may be obtained in the presence or absence of added water. Previous results may have arisen from the accidental presence of seed crystals or of traces of acid catalysts (22).

The products obtained by treating galactose with liquid ammonia or 30 per cent ammonium hydroxide were analyzed after pressure reduction with nickel catalyst (23). The products apparently obtained are shown in Formulas IV, V, VI, VII, and VIII.

$$
\begin{array}{ccccc}
\text{CH}_2\text{NH}_2 & \text{CH}_2\text{NH}_2 & \text{CH}_2\text{OH} & \text{CH}_2\text{—NH—CH}_2 & \text{CH}_2\text{NH}_2 \\
| & | & | & |\qquad\qquad| & | \\
(\text{CHOH})_4 & \text{CHNH}_2 & (\text{CHOH})_4 & (\text{CHOH})_4\ (\text{CHOH})_4 & \text{CH}_2\text{NH}_2 \\
| & | & | & |\qquad\qquad| & \\
\text{CH}_2\text{OH} & (\text{CHOH})_3 & \text{CH}_2\text{OH} & \text{CH}_2\text{OH}\ \ \text{CH}_2\text{OH} & \\
& | & & & \\
& \text{CH}_2\text{OH} & & & \\
\\
(\text{IV}) & (\text{V}) & (\text{VI}) & (\text{VII}) & (\text{VIII})
\end{array}
$$
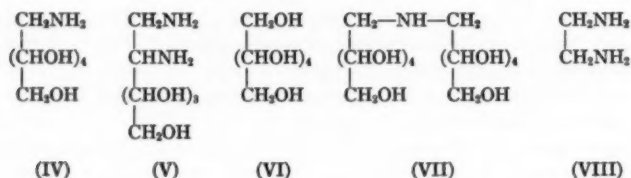
All had the D-galactose configuration, and all but the 1,2-dideoxy-1,2-diamino-D-galactitol were isolated as crystalline derivatives. Schiff bases formed from aromatic aldehydes (benzaldehyde and salicylaldehyde) seem to be quite satisfactory for the isolation of these amino compounds. The N-benzylglycosylamines seem especially suited for the preparation of 1-amino-1-deoxy-glycitols (glycamines) into which they are converted in good yields (50 to 65 per cent) by pressure hydrogenation.

The potassium salts of glucuronic acid readily form unstable glycosylamines when reacted with aromatic amines (24). Such derivatives are metabolites of the cancerigenic 2-naphthylamine (25). Mammalian livers contain enzymes associated with the microsomes which transfer glucosyluronic acid residues from uridine diphosphate glucuronic acid to aromatic amines (26). Some microorganisms excrete an anthranilic acid derivative which seems to be an intermediate in the synthesis of indole and tryptophan (27, 27a). The derivatives may be a fructosylamine or an Amadori rearrangement product of the ribosylamine.

Great interest has been shown in the synthesis of analogues of natural nucleosides, especially those with unnatural sugars. The usual purpose has been a search for biological antagonists of nucleoside metabolism and for antitumor agents. Such compounds are usually covered in other chapters of this review. An example is afforded by the nucleosides derived from 6-D-allofuranose, which, however, were devoid of antitumor activity (28).

THE AMADORI REARRANGEMENT

The glycosylamines react readily in solution or even in the solid state. In addition to undergoing anomerizations, ring changes, and hydrolysis, they may undergo the Amadori rearrangement in which aldosylamines isomerize to ketose derivatives (see Formulas IX and X).

$$
\begin{array}{ccc}
\text{HC—NHR} & & \text{H} \\
| & & | \\
\text{HCOH} & O & \text{HC—NHR} \\
| & & \text{C=O} \\
& & |
\end{array}
$$

(IX) Glycosylamine   (X) 1-Amino-1-deoxyketose
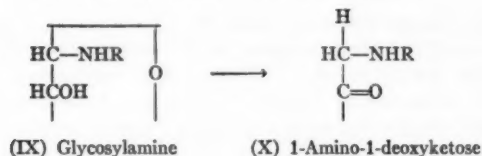
The general characteristics of the reaction have been described by Hodge (29), who also points out that the action of amines on sugars has many analogies to the action of alkalies. The products formed from amines, however, provide stable intermediates that may be useful in studying such reactions. Interestingly, products analogous to the saccharinic acids have not been described in rearrangements of glycosylamines.

The reaction offers the intriguing possibility that it may stabilize the linkage between sugars (or polysaccharides) and amino compounds, especially proteins, since the reaction appears usually to be irreversible. Amorphous compounds of this type have been isolated from liver and microorganisms (27a) but have not been shown to be a linkage in glycoproteins. Micheel & Frowein (31) obtained crystalline derivatives of glycine (and other amino acids) by using 4,6-O-benzylideneglucose and the esters of the amino acids.

Fructose reacted directly with amino acids (glycine, alanine, and leucine) to give fructosylamines which isomerized to the substituted 2-amino-2-deoxy glucose (32, 32a). The glycine derivatives also gave the corresponding mannosamine derivative. Although usually the Amadori rearrangement is irreversible, these products were converted to fructose and amino acid by aqueous mineral acids. However, the 1-deoxy-1-*p*-toluino-lactulose also was said to be hydrolyzed by acids (32b).

In possible analogy to some reactions with proteins, D-glucose condensed with polyvinylamine to give N-derivatives of 1-amino-1-deoxy-D-fructose (33). The product may undergo a secondary condensation between the reducing group of the fructose derivatives and other amino groups (Formulas XI and XII).

$$
\begin{array}{ccc}
\text{—CH}_2\text{—CH—CH}_2\text{—CH—} & & \text{—CH}_2\text{—CH—CH}_2\text{—CH—} \\
| \quad\quad | & & | \quad\quad | \\
\text{NH} \quad\quad \text{NH}_2 & & \text{NH} \quad\quad \text{NH} \\
| & & | \quad\quad | \\
\text{HCH} & & \text{CH} \text{———} \text{C} \\
| & & | \\
\text{C=O} & & O \\
| & & |
\end{array}
$$

(XI)   (XII)
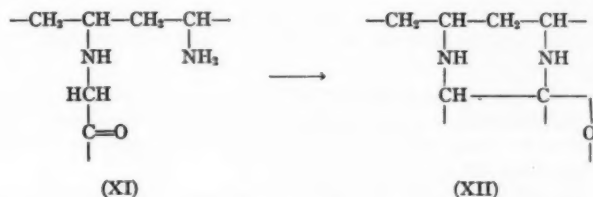
The glycosylamines derived from glucuronic acid and aromatic amines underwent the expected Amadori rearrangement in the presence of acetic acid and gave crystalline products (24).

Although Hodge (29) suggested that the Amadori rearrangement requires both acid and basic catalysis, the necessity of base catalysis has only been recently demonstrated for N-*p*-tolyl-D-glucosylamine. The isomerization of this substance to 1-deoxy-1-*p*-toluino-D-fructose under the fusion conditions of Weygand proceeded easily in the presence of acid catalysts but not in methanol solution. With pyridine as the solvent, the reaction went well (34). The presence of the salts of organic acid or some phenols in methanol also improved the yield considerably in comparison with the action of the acid alone (35).

With N-*p*-tolyl-D-glucosylamine, the Amadori rearrangement was accompanied by three other reactions, two leading to colored materials (35, 35a). Maximum yields were obtained when these reactions were suppressed. These results indicate that many presumed instances of a lack of Amadori rearrangement may be the result of destruction of the reaction product rather than to no reaction.

The effect of aromatic substitution on the formation of 1-N-aryl-1-deoxy-D-fructose from D-glucose and substituted anilines has received some preliminary study (35, 36) under the fusion conditions of Weygand with HCl as catalyst.[4] The two studies are not in complete agreement since Micheel & Schleppinghoff (36) reported no rearrangement of N-*p*-chlorophenyl-D-glucosylamine, whereas Rosen, Woods & Pigman (35) found some rearrangement of this compound and its N-*p*-bromo analogue. The reaction apparently did not proceed with the glycosyl derivatives of weak amines such as *p*-nitroaniline. As indicated by Micheel & Schleppinghoff (36), the rearrangement apparently requires an increase in the electron density at the carbon atom attached to the nitrogen atom, and this would be achieved by the addition of a proton to the nitrogen atom. Micheel & Schleppinghoff (36), however, found that a number of meta substituted anilines did not undergo the reaction; especially with the *m*-toluidine derivative, the ease of rearrangement did not seem to parallel the basicity of the amines.

The apparent difficulty of rearranging N-alkyl-glycosylamines may arise from the tendency of these substances to form diglycosylamines. Thus, such derivatives of secondary alkyl amines, e.g., di-*n*-butylamine and dibenzylamine, undergo the rearrangement (29) and will not form diglycosylamines.

The reduction of the rearrangement product of N-benzyl- and N-arylglycosylamines provides a new method of preparing amino sugars (37, 37a). The 1-deoxy-1-dibenzylamino-D-fructose gave 1-amino-1-deoxy-D-fructose in 94 per cent yield (37a).

The so-called "reverse" Amadori rearrangement of fructosylamines to 2-deoxy-2-amino-D-aldoses has received additional study (16). The method has value especially for the preparation of 2-deoxy-2-amino aldoses. When

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[4] Professor Micheel informs us that the results reported in Table 1 of (36) were for fusion conditions and not for ethanol solutions.

the reaction was carried out with fructose and ammonia in the presence of ammonium chloride (Formula XIII), yields of 12 per cent were obtained.

$$\begin{array}{ccc}
CH_2OH & CH_2OH & HCO \\
| & | & | \\
C=O + NH_3 + NH_4Cl \rightarrow & C-NH_2 \rightarrow & HC-NH_2 \\
| & |\diagdown & | \\
 & O \\
 & |
\end{array}$$
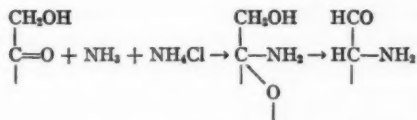
(XIII)

The yields can be about doubled if oxalic acid is used as the catalyst in the rearrangement stage. Although D-fructose yielded mostly D-glucosamine, and D-tagatose mostly D-galactosamine, L-sorbose and D-allulose gave mixtures of the epimeric amino sugars.

In the Amadori rearrangement of D-fructosylamine, formic acid, succinic acid, and benzoic acid were classified as good catalysts and stronger (trichloroacetic acid) and weaker acids (acetic acid) as poor catalysts (16). These results are similar to those described above for N-p-tolyl-D-glucosylamine except that acetic acid was an effective catalyst for the rearrangement from aldose to ketose. Probably better yields would have been obtained by the use of an added basic catalyst such as the salts of the acids.

Ketosylamines derived from aromatic amines did not undergo the rearrangement (16), as had also been previously observed. This result presents an interesting contrast to the aldosylamines for which those derived from primary aliphatic amines apparently do not react under the usual conditions.

Amadori rearrangements which do not proceed under ordinary conditions may occur in the presence of "active methylene" solvents (29). Such solvents are undoubtedly weaker proton donors than phenol, which is not a satisfactory catalyst (35). Unless an entirely different mechanism is involved, the most likely explanation may be a general "solvent" effect similar to that for pyridine, which in low concentrations has no catalytic action (35). Hydrogen bonding at a number of centers in the reacting molecule may have a general electron-repelling or attracting action with an over-all effect similar to that of a stronger acid or base attacking one center.

Since many glycosylamines readily form osazones in high yield under the usual conditions of osazone formation, the Amadori rearrangement has usually been assumed to be a step in the conversion of hydrazones to osazones. D-Glucose-1-T, however, was converted to its phenylosazone without loss of tritium (38). The key step is:

$$\begin{array}{ccc}
H \\
T-CNH-NHPh & \longrightarrow & T-C=N-NHPh \\
| & & | \\
C=O & & C=NH-NHPh \\
| & & |
\end{array}$$

(XIV)

One hydrogen is lost at C-1, and some tritium would be expected to be lost despite an obvious isotope effect. Weygand, Simon & Klebe (39) found, however, that a marked isotope effect exists in this reaction and that the corresponding reaction with D-glucose-1-D,T freed some tritium. When D-glucose was used and the reaction was carried out in tritiated water, a strong uptake of tritium (48 per cent)occurred during the reaction.

NOMENCLATURE OF CARBOHYDRATE-PROTEIN INTERACTION PRODUCTS

The nomenclature of compounds or complexes containing carbohydrates and proteins has been reviewed by Pigman & Platt and a modified system suggested (3). This system will be used in the present review and differs mainly from that of K. Meyer in that the basic older significance of glyco-proteins will be retained as firmly bound combinations of saccharides and polypeptides, both present in substantial amounts, and usually with a covalent bond.

Mucoids probably should be considered as a type of glycoprotein. If the term is used, it should probably mean a water-soluble glycoprotein which does not precipitate on the addition of weak acids unless free protein is present.

In agreement with K. Meyer, mucoproteins are dissociable complexes of proteins and carbohydrates (or glycoproteins). Some animal mucins, at least, belong to this group. The mucin of synovial fluid, precipitated by weak acids, is a dissociable complex of albumin and hyaluronic acid (40).

Although badly needed, basic improvements in the nomenclature are not yet possible and must await further knowledge in the field.

LINKAGES IN GLYCOPROTEINS AND MUCOIDS

In attempts to elucidate the structure of glycoproteins, the location of sialic acid in cattle submaxillary mucin has been most extensively investi-gated (41 to 44). Evidence has accumulated that sialic acid is present in the mucin in a terminal position and that its reducing group is engaged in a glycosidic bond, with galactosamine to form disaccharide side chains to a peptide.

Similar results have been obtained for orosomucoid (45, 46) prepared from nephrotic urine (47). The receptor-destroying enzyme from *Clostri-dium perfringens* liberated all the sialic acid, and this liberation was ac-companied by an increase of reducing power in the dialyzable fraction (sialic acid) and by a decrease of acid groups in the nondialyzable residue. Periodate acted on the orosomucoid with a time curve that indicated two types of reaction (46). A rapid reaction was found associated with sialic acid, and a slow reaction with the nondialyzable portion. The bonds linking sialic acid and fucose to the orosomucoid were quite sensitive to acid. The hexose and hexosamine were released much more slowly (48).

The preparation and characterization of a series of oligosaccharides by mild hydrolysis have also been an important approach to the elucida-

tion of the detailed structure of several other glycoproteins. The 4-O-β-D-galactopyranosyl-2-acetamido-2-deoxy-D-glucose (N-acetyllactosamine) has been prepared from blood group substances of hog gastric mucin (49, 50, 51) and of meconium (52). Acetolysis of blood group mucoid from hog gastric mucus and subsequent deacetylation have given two oligosaccharides, O-2-acetamido-2-deoxy-D-galactopyranosyl-(1→4)-O-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-D-glucose (53) and 4-O-[2-acetamido-2-deoxy-α-D-galactopyranosyl]-D-galactose (54). When purified human Group A substance from ovarian cyst fluid was subjected to partial acid hydrolysis, four nitrogen-containing disaccharides besides N-acetyllactosamine were obtained (55). One proved identical with 3-O-β-D-galactopyranosyl-2-acetamido-2-deoxy-D-glucose isolated by Kuhn *et al.* (56) from human milk. For the remaining three disaccharides, which have not yet been firmly characterized, the structures of 3-O-[2-acetamido-2-deoxy-β-D-glucopyranosyl]-D-galactose, 3-O-[2-acetamido-2-deoxy-α-D-galactopyranosyl]-D-galactose and 6-O-L-fucosyl-2-acetamido-2-deoxy-D-glucose, respectively, have been suggested on the basis of their chromatographic behavior and general properties (55).

Very little is known of the nature of the bonds of glycoproteins. Johansen, Marshall & Neuberger (57) obtained a peptide-carbohydrate complex (glycopeptide) from egg albumin. Successive proteolysis with pepsin, trypsin, and chymotrypsin, and mold protease was followed by column chromatography on charcoal-Celite and by column electrophoresis. The complex was shown to contain mannose, glucosamine, leucine, and aspartic acid in a molar ratio of 5 : 3 : 1 : 1. Small amounts of serine and threonine were also detected. After prolonged dinitrophenylation, dinitrophenyl-aspartic acid was obtained. Digestion of the complex with carboxypeptidase liberated all the amino acids except aspartic acid. These observations suggested that the aspartic residue was directly linked with carbohydrate through one of its carboxyl groups. Glycopeptides of similar composition have also been prepared from the same source by Cunningham, Nuenke & Nuenke (58) and by Jevons (59).

After proteolysis of human γ-globulin, three glycopeptides, two of which were probably the degraded products of the other (glycopeptide I), were also obtained (60). The molar proportion of the glycopeptide I was: hexose, 8.3 (galactose : mannose, 3 : 5), glucosamine, 6.0, fucose, 2.0, sialic acid, 1.0, aspartic acid, 2.1, glutamic acid, 3.3, and tyrosine, 0.95. Further digestion of the glycopeptides by means of leucine aminopeptidase, aminopeptidase and carboxypeptidase revealed that one aspartic residue was at the C-terminal position, probably linked with a carbohydrate residue through the β-carboxyl group.

Aspartic acid is thus the most likely amino acid residue linked to carbohydrate in two glycopeptides of different origins (57, 60). The fairly high yields of these glycopeptides (59, 60) suggest that an ester or amide bond may be the main chemical bond to link carbohydrates to amino acids in

natural glycoproteins. However, the presence of free C-1 amino acid derivatives of fructose in hog liver also suggests the presence in glycoprotein of such bonds formed from glucosylamines by an Amadori rearrangement (30).

CHONDROMUCOPROTEIN

The early study by Shatton & Schubert (61) demonstrated that chondroitin sulfate A existed in cartilage as a complex of protein, called chondromucoprotein. The chondromucoprotein prepared from cattle nasal cartilage has been shown to contain 5.15 per cent nitrogen and 17.4 per cent hexosamine (62). If the cartilage is completely disintegrated, approximately 80 per cent of the hexosamine present can be extracted with water as the mucoprotein (62). Mucoproteins of the same type but of somewhat different composition have also been isolated from horse nasal (63) and pig tracheal cartilage (64). The mucoprotein from pig tracheal cartilage was shown (64) to contain 3.92 per cent nitrogen and 27.4 per cent hexosamine and to migrate as a single boundary in electrophoresis over a range of pH 4 to 7.4 and of ionic strength 0.2 to 0.8.

The chemical nature of the protein moiety of the chondromucoprotein from cattle and horse nasal cartilage and pig tracheal cartilage has been extensively investigated (63 to 67). The amino acid composition was markedly different from that in collagen, and no hydroxyproline could be found. Collagen coextracted with the mucoprotein could be removed by treatment of the extract with a carboxylic acid ion exchange resin (65).

Digestion with pepsin (64), trypsin (61, 68), or papain (64) reduced the viscosity of the chondromucoprotein solution considerably. The pure polysaccharide could be isolated easily from these digests (61, 64). Relatively weak alkali could also bring about a similar effect on the viscosity of the mucoprotein solution (64). Crystalline barium chondroitin sulfate A was obtained in fairly good yield after treatment of the mucoprotein with 0.18 M sodium hydroxide at 37°C. for 20 hr. (62). The carboxylic acid resin adsorbed the protein moiety of the mucoprotein only when the mucoprotein was previously treated with alkali (65). Electrophoretic study of the mucoprotein from cattle nasal cartilage over the pH range 2 to 12.5 revealed that the mucoprotein was dissociated irreversibly at pH 12.5 into two components, one of which had the same mobility as chondroitin sulfate A (68a).

The protein moiety of the complex from pig tracheal cartilage contained a large amount of serine (64). This amino acid has also been found most difficult to remove from the polysaccharide by treatment of the complex with papain. On the basis of these observations and of the cleavage of the linkage by alkali, Muir suggested an ester linkage through the hydroxyl group of serine for this complex (64). However, the possibility of a salt-type linkage with a strongly basic protein has not been eliminated.

The protein content of the chondromucoproteins is rather low, so that the removal of the protein should not greatly affect the viscosity unless it joins together longer carbohydrate chains (64). The molecular weight of the chondromucoprotein from bovine nasal cartilage has been found to be

about one million (66, 67, 68). This value is extremely high as compared with the molecular weight of 5×10^4 for isolated chondroitin sulfate A (69). After digestion with trypsin, the molecular weight of the mucoprotein decreased from 1.0×10^6 to 3.9×10^4, which is comparable with the value for pure chondroitin sulfate A (68). These various observations suggest that the complex consists of a number of the polysaccharide molecules bound together with polypeptide chains mainly with end-to-end arrangement (63, 64, 66, 68). An investigation with the electron microscope of the chondromucoprotein from aqueous solutions showed the presence of a fibrous structure (63). Mathews & Lozaityte (67), however, find that the physical data fit better a protein core covered with the smaller chondroitin sulfate molecules, which can be removed by hyaluronidase treatment.

L-IDURONIC ACID-CONTAINING POLYSACCHARIDES

L-Iduronic acid is a component of a polysaccharide found widely distributed in animal tissue, especially skin (70). Apparently the same material was identified by Aizawa (71) as containing D-galacturonic acid. Various names have been given, such as "β-heparin," "chondroitin sulfate B," "dermoitin sulfuric acid," and "gastroitin sulfuric acid." Since a principal source is skin, the compound will be described here as "derman sulfate."[5]

The identification of L-iduronic acid has been further substantiated (72, 73). Derman sulfate was desulfated and reduced by sodium borohydride; hydrolysis gave L-idose, subsequently transformed into L-idosan (72), and evidently arising from L-iduronic acid.

A small amount of glucuronic acid had also been detected in such hydrolyzates (70). The glucuronic acid appeared to originate from another polysaccharide present in the purified derman sulfate (β-heparin) fraction (73, 74). This polysaccharide is different from chondroitin sulfates A and C, as indicated by its optical rotation and resistance to testicular hyaluronidase (73, 74).

Testicular and pneumococcal hyaluronidase did not attack derman as well as the original derman sulfate (chondroitin sulfate B) (74).

Derman sulfate hydrolyzed more easily than hyaluronic acid or chondroitin sulfate A. Hydrolysis with 1N HCl for 1 hr. produced approximately 50 per cent of monosaccharides and 50 per cent of oligosaccharides. The purified oligosaccharide fraction gave a product with an absorption maximum at 510 mμ when tested with Elson-Morgan reaction. Since this maximum is characteristic for a 3-substituted hexosamine (75), a uronosyl-(1→3)-hexosamine structure was suggested (73).

Derman sulfate could be fully methylated under suitable conditions (76). Methanolysis of the methylated product followed by N-acetylation gave

[5] This name was suggested by Dr. Roger Jeanloz (private communication) along with "dermatan sulfate." Since polysaccharides generally are indicated by the "an" ending, e.g., "xylan," this ending is much preferable to "in." The use of "tan" seems superfluous and not in accord with general usage except in this special field.

crystalline methyl 2-acetamido-2-deoxy-6-O-methyl-α-D-galactopyranoside (72, 76). On the other hand, methylation of the derman (after desulfation) led to crystalline methyl 2-acetamido-2-deoxy-4,6-O-dimethyl-α-D-galacto-pyranoside (74). These data indicate the position of the sulfate group to be at C-4 of the galactosamine, and, accordingly, the L-iduronidic linkage to be at C-3. This conclusion is also supported by infrared analysis (77).

These infrared studies by Mathews (77) also indicate that the sulfate group is at C-4 for chondroitin sulfate A, and at C-6 for chondroitin sulfate C. The 4-sulfates are in axial positions, and the 6-sulfate has an equatorial position.

HEPARITIN SULFATE

The isolation and characterization of a sulfated polysaccharide, related to heparin and named heparitin sulfate or heparin monosulfate, have been described. Although this polysaccharide was also found in amyloid liver and in certain normal tissues, such as human and cattle aorta (78, 79), most isolation studies have been made on the urine (78 to 81), liver (78, 79, 81, 82, 83) and spleen (82, 83) of patients with gargoylism.

Heparitin sulfate contains one sulfate group per disaccharide unit (79, 80, 82). Like heparin, the compound gave a very high uronic acid value by the Dische carbazole method and showed an unusual dextrorotation (79 to 82). It was resistant to testicular, bacterial, and leech hyaluronidase (79, 80) but was hydrolyzed by enzymes obtained from a flavobacterium adapted to heparitin sulfate. These enzymes could also hydrolyze heparin (79).

Unlike heparin, heparitin sulfate contained an acetyl group, passed through a cellophane membrane (although at a very slow rate), and had negligible anticoagulant activity (82). The molar ratio of acetyl to nitrogen was approximately 0.5. Desulfation experiments showed that some of the sulfate groups were present in a sulfamide linkage to the nitrogen of the hexosamine residue.

The polysaccharide seems to be identical to the heparin monosulfuric acid prepared from ox liver or lung by Jorpes & Gardell (84) in 1948. The infrared spectra of the heparitin sulfate fractions obtained from gargoylism liver and ox lung have been shown to be identical (79).

Heparitin sulfate seems to be structurally similar to heparin, but the molecular size is smaller, some of the amino groups carry acetyl groups, and the percentage of sulfate groups is smaller (79, 80, 82).

ANIMAL MUCUS AND MUCINS

No basic chemical similarities for the various types of mucus and mucins have been established, since the compositions have received so little study. Their physical behavior as clear, viscous, stringy solutions derived from animal tissues is their principal common basis, although sometimes mucin clot formation by weak acids provides the basis (3). Histologically, the mucins (or mucus) may be the secretions on mucous membranes with metachromatic staining properties or oxidizable by periodic acid. The relationship to the "ground substance" of tissues has not been clarified.

Synovial mucin.—As shown by electrophoretic studies by Pigman *et al.* (40), the mucin of human synovial fluid is a mucoprotein composed principally of hyaluronic acid and albumin, in the approximate ratio of 1 : 4. However, Blumberg & Ogston (85) continue to provide evidence that the hyaluronic acid is loosely associated in cattle (and human) synovial fluid with a protein, probably an α-globulin, which is said to comprise 25 per cent of the mucin.

Salivary mucins.—Of the mucins of salivary extracts, the carbohydrate portion of cattle, sheep, and pig submaxillary mucins have received some recent study. According to Heimer & Meyer (42), a purified cattle mucin preparation contained 25 to 30 per cent of sialic acid, 19 per cent of hexosamine, and a trace of hexose and L-fucose. Odin (86) obtained products with similar analyses from cattle and sheep submaxillary mucins, although prepared according to McCrea (87) by a more direct fractionation of the extract. These mucins contained four to five times as much galactosamine as glucosamine, whereas hog mucin had only galactosamine.

The L-fucose of human whole salivas and submandibular secretions was considered by Berggård & Werner (87a) to be a component of the mucin.

Epithelial mucins.—Following Blix and Werner, Odin (86) classified the glycoproteins found in epithelial mucus into two main groups, the fucomucins and sialomucins, which may often occur together as in human saliva (87a). The fucomucins are relatively rich in L-fucose and contain N-acetylglucosamine, N-acetylgalactosamine and D-galactose. Some fucomucins contain a small amount of sialic acid. Well-known fucomucins are the glycoproteins with blood group activity obtained from the pseudomucinous ovarian cyst fluid and pig gastric mucus. To the sialomucins belong the glycoproteins found in mucus such as saliva and hog seminal gel. In the sialomucin, the sialic acid is accompanied by an equivalent amount of N-acetylhexosamine, usually galactosamine.

The epithelium of the oviducts of the hen produces a secretion containing the so-called ovomucoid and ovomucin which are quite different from the fuco- and sialomucin. The ovomucoid was composed of 14 to 15 per cent glucosamine, 9 per cent hexoses (galactose 0.33, mannose 1) and 1 per cent sialic acid; the ovomucin contained 7.3 to 7.5 per cent hexosamine (glucosamine 3, galactosamine 1), 7 to 8 per cent hexoses (galactose 2 to 3, mannose 1), and 6 to 7 per cent sialic acid (86).

Masamune & Tsuiki (88) isolated from pig stomach mucosa four polysaccharides which contained hexosamines, sialic acid, L-fucose, and hexoses. Two were of the sialomucin type without blood group activity. One may correspond to Castle's intrinsic factor. The two other mucopolysaccharides showed Group A activity strongly, O activity weakly, and were similar to the fucomucins of Odin.

Snail mucins and polysaccharides.—From the albumin glands of the Roman snail, *Helix pomatia*, Geldmacher-Mallinckrodt and May (89 to 92) isolated new polysaccharides closely related to the older described sinistrin or galactogen. Analysis of two of these showed that they differed from sinis-

trin in that they were not precipitated with an alkaline copper solution and stained only faintly after treatment with periodic acid-parafuchsin. One had only a small content of L-galactose, had a strongly negative rotation, and was rich in hexose phosphate. The other was richer in L-galactose and also had an appreciable amount of hexose phosphate.

Kwart & Shashoua (93, 94) investigated the mucus secreted by a marine snail, *Busycon canaliculatum L.* They suggested a rather improbable structure of a complex of protein and polysaccharide combined through salts such as $CaSO_4$ or Na_2SO_4. The polysaccharide was said to be composed of only hexosamine units, the amino group of which was combined as sulfate salts. The mucus was very viscous, and had a viscosity of 60 to 100 centipoises at 0.3 per cent organic solids. Its characteristic properties were correlated with a spherically shaped molecule of polyelectrolyte nature.

A polysaccharide obtained from palmonate aquatic snails also was analyzed (95). It contained galactose, fucose, glucosamine, and a few unidentified substances. (Galactose and fucose are often not distinguished as D or L in such work, although both forms are known in such products.) Females of two species of operculate snails did not contain true galactogen but contained a galactose-fucose polysaccharide and glycogen, whereas males contained only glycogen. Egg clutches were also analyzed.

Fish mucins.—Wessler & Werner (96) investigated the composition of mucus from fishes such as cod, whiting, ray, and eel. The principal component of the external surface mucus seemed to be a simple protein, sometimes mixed with small and varying amounts of nucleic acid and glycoproteins. The latter contained hexosamine, galactose, fucose, and sialic acid. The fish-roe mucus contained hexosamine, galactose, mannose, and sialic acid in addition to ribose, fucose and/or glucose. The carbohydrate moiety probably forms a substantial part of the jelly coat of the roe.

ANALYSIS OF MATERIALS CONTAINING HEXOSAMINES, URONIC ACIDS AND SIALIC ACIDS

Methods for the analysis of carbohydrate materials have been described in a number of compilations (3, 97, 98a, b, c, d).

Uronic Acids.—Uronic acids are frequently determined satisfactorily by the Dische carbazole method (98a) or by the Tracey carbon dioxide method (99).

Dische points out that destruction of monosaccharide units during the hydrolysis of polysaccharides or glycoproteins is an important source of error (98a). Hydrolysis with a sulfonated polystyrene resin offers some advantages for the liberation and subsequent determination of glucuronic acid in tissue samples (100). Hexosamine and hydroxyproline could also be determined in the same hydrolyzate. Masamune, Sakamoto & Aizawa developed conditions for the determination of glucuronic acid and galacturonic acid in the presence of one another (101, 102). These determinations take about two days to complete, require fairly rigid control of conditions, and are not very sensitive.

A procedure for tentatively identifying galacturonic acid (on paper chromatograms) in the presence of glucuronic acid depends on partially lactonizing glucuronic acid before chromatography (103). The identification is accomplished by characteristic migration rates, hydroxamic acid-ferric ion test for lactones and a specific lead acetate test for galacturonic acid.

Glucuronic acid (104) has also been determined by use of naphthoresorcinolcarboxylic acid. Glucose and ascorbic acid interfered to only a minor extent. Glucose, glucuronic acid, and ester glucuronate were oxidized by bromine to give nonreactive products. Glycosiduronic acids, whose blocked aldehyde group was not oxidized under these conditions, could then be estimated.

The Dische carbazole method (105) gives different color intensities for the various uronic acids (72). An orcinol method used by Khym & Doherty (106) shows less difference (72), but hexoses and pentoses interfere more.

In another application of the Dische carbazole method, glucuronic acid, glucose, and xylose can be estimated in mixtures (107), specifically those in acidic polysaccharides and in urine. When xylose was present, the accuracy of the method was considerably reduced. Recoveries of glucurone and glucose added to urine were good and were not affected by the addition of albumin, ketone bodies, urea, or arabinose in limited quantities. Analyses for uronic acid in chondroitin sulfate, hyaluronic acid and heparin agreed with other methods.

Mixtures of galacturonic acid, galactose, and rhamnose can be estimated simultaneously by the anthrone reaction (108). With this method oligogalacturonides gave absorbances equivalent to galacturonic acid. Heparin had an absorbance much greater than that of an equivalent amount of glucuronic acid. Dische (105) and then Bowness (107) had reported similar results for the carbazole reaction. A naphthoresorcinol reaction, in which butyl acetate was used to extract the coloring matter, has been applied to the determination of glucuronic acid in plasma, blood, and urine (109).

5-Formylfuroic acid was identified as the main chromogen in the reactions of uronic acids in sulfuric acid (110). Its formation is analogous to that of hydroxymethylfurfural and furfural from hexoses and pentoses under similar conditions.

Hexosamine determinations.—N-Acetylhexosamines are generally determined by reaction with alkalies and then in acid medium with *p*-dimethylaminobenzaldehyde (Morgan-Elson reaction). Hexosamines are determined similarly after alkaline condensation with acetylacetone (Elson-Morgan reaction). The many modifications of the methods which have appeared are an indication of the difficulties of obtaining satisfactory results, although with extreme care the methods are sometimes satisfactory. Probably, however, a new type of method is needed.

Gardell (98b) has recently reviewed these methods. The modifications by Blix (111) and Boas (112) seem to be used most often for hexosamines, and the modifications of Aminoff, Morgan & Watkins (113) and Reissig, Strominger & Leloir (114) for acetylated hexosamines. A recent procedure of

Svennerholm (115) seems to offer advantages. Kraan & Muir (116) were able to double the sensitivity of Rondle & Morgan's modification (117), an already sensitive procedure.

An ultramicro method (118) based on the earlier procedure of Dische and Borenfreund involved deamination with nitrous acid and reaction of the 2,5-anhydrosugar with pyrrole. D-Xylosamine behaved similarly to glucosamine in the Dische-Borenfreund method (119).

The Elson-Morgan modification of Boas (112) has been used to determine the position of substitution of hexosamines in several zoöpolysaccharides (74). The 3-substituted hexosamines gave colored solutions with an absorption maximum at 510 mμ and 4- and 6-substituted hexosamines showed maxima at 535 mμ. The method was used to show the presence of 3-substituted hexosamines in keratosulfate, derman sulfate, and hog gastric mucin. No shift in the absorption maximum was obtained when the Morgan-Elson method was applied to similar substances, but the absorbance differed markedly with the position of substitution (120, 121).

Glucosamine and galactosamine are usually determined in mixtures by adsorption on ion-exchange resins and stepwise removal by the Gardell method (98b). Baker's yeast, usually reported as not fermenting D-glucosamine, will remove it when present in large quantities. This observation provides a sensitive method for the analysis of such mixtures (122).

D-Talosamine has been separated from glucosamine and galactosamine by use of columns of Zeo-Karb 225 resin (123). The talosamine was obtained from a sample of recrystallized N-acetylgalactosamine originally derived from tracheal cartilage and apparently was an artifact.

The nature of the chromogens formed in the Elson-Morgan and Morgan-Elson methods has been clarified. White's early identification of an oxazoline in the products formed by the condensation of N-acetyl-D-glucosamine with alkali (Morgan-Elson reaction) now seems incorrect (124). Kuhn & Kruger (125) obtained a 40 per cent yield of 5-dihydroxyethyl-3-acetamidofuran XV. A 3-acetamidofuran was also obtained by the vacuum distillation of N-acetylglucosamine (125, 126).

CH₂OH—CHOH—[====]—NHCOCH₃   [====]—CH₃
              O   N
                                         H
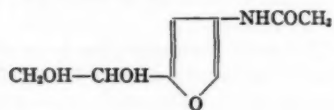
(XV) Intermediate in   (XVI) Intermediate in
Morgan-Elson Reaction   Elson-Morgan Reaction

The principal corresponding product formed by the alkaline condensation of acetylacetone with glucosamine was identified (127) as 2-methylpyrrole XVI accompanied by some 3-acetyl-2-methylpyrrole.

Sialic acids.—Svennerholm (128) investigated the optimal conditions for the determination of sialic acids with orcinol-HCl (Bial's Reagent). Cupric

ions were at least as effective as ferric ions in increasing the sensitivity of the reaction. Interference from other carbohydrates was eliminated by reading at two wavelengths. A new method (129) of determining sialic acids using resorcinol-HCl was found to be 50 per cent more sensitive than orcinol-HCl (128), and less interference from carbohydrates resulted.

The lack of specificity of color reactions (128, 129, 133) for sialic acids has necessitated their separation from other components before analysis. Svennerholm (130) used Dowex-2 (acetate form) to separate sialic acids in hydrolyzates and then determined them by the resorcinol method (129). N-Acetyl and N-glycolylneuraminic acids have been quantitatively separated by paper chromatography using *n*-butanol : *n*-propanol : O.1N HCl (1 : 2 : 1, v/v) (131). Other forms of sialic acids were not separated by this solvent system. This method is more direct than that of Klenk & Uhlenbruck (132), who developed a micromethod for determining glycolic acid in isolated sialic acids and, thus, the N-glycolylneuraminic acid content.

Pigman *et al.* (133) have suggested the concurrent use of two methods for determining sialic acid. In this way marked discrepancies between the methods may indicate the presence of interfering materials or of different forms of sialic acids.

β-Glucosidases, Cellulases, and Biosynthesis of Cellulose

Despite the great industrial importance and wide distribution of cellulose, relatively little has been known of the biosynthesis (133a). Although usually considered only of interest in plant materials, cellulose is formed by some microorganisms and lower animals (Tunicata). Recent claims have been made of the occurrence of cellulose fibers in a mammalian tissue, bovine skin (134). This important observation needs confirmation and assurance that the cellulose is not an artifact.

Cellulose Synthesis.—Using the fresh cells of *Acetobacter xylinum*, Greathouse (135) found that the distribution of C^{14} in the glucose units of cellulose produced from D-glucose-6-C^{14} was approximately 82 per cent of the label in the original position 6, and 9 per cent was in position 1. The distribution of C^{14} in the glucose units formed from glycerol-1,3-C^{14} was approximately 12, 4, 22, 29, 3 and 30 per cent at carbons 1, 2, 3, 4, 5 and 6, respectively. A cell-free particulate enzyme system which was capable of synthesizing cellulose-C^{14} directly from D-glucose-1-C^{14} and ATP was also obtained from this bacterium (136). With this system, 96 per cent of the label was found at C-1 of the glucose residues of cellulose-C^{14}. These results suggested that most of cellulose was synthesized directly from glucose without cleavage of its carbon chain, possibly through hexose phosphate, but some of cellulose was apparently resynthesized from the cleavage products of hexose such as glyceraldehyde.

Schramm, Gromet & Hestrin (137) used nonproliferating washed cells or freeze-dried cells of *A. xylinum.* They found an oxidation process involving the pentose cycle to be indispensable for the cellulose synthesis. Apparently, through this cycle some hexose phosphate is supplied for cellu-

lose synthesis. As a result, cellulose synthesis decreased when the bacterium was active metabolically (138). However, the phosphate esters [including glucose-6-phosphate, α- and β-glucose-1-phosphate and uridine diphosphate glucose (UDPG)], when added exogenously to fresh and freeze-dried cells, did not give rise to cellulose, whereas glucose, fructose, glycerol, dihydroxy-acetone and hexonates were all converted into cellulose. Presumably the phosphate esters did not penetrate into the cells. Cellulose could not be formed from any of substrates tested with any cell-free extract (139).

Glaser (140) synthesized cellulose from UDPG-C[14] and primer cello-dextrins by using a cell-free extract of *A. xylinum*. The extract contained a particulate enzyme system which was obtained by rupturing cells with a magnetic oscillator. The closely similar chitin was synthesized also by Glaser & Brown (141) from uridine diphosphate N-acetylglucosamine by using a pellet extracted from the mycelium homogenates of *Neurospora crassa*. Chitodextrin was needed as primer.

Feingold, Neufeld & Hassid (142) found that extracts from the seedlings of mung bean (*Phaseolus aureus*) and other several higher plants could catalyze the transfer of D-glucose from UDPG to form a β-1,3-linked glucan. The action of the enzyme system was stimulated by D-glucose and a number of D-glucosides.

Cellulases.—Celluloses are hydrolyzed by enzymes from various sources. Using the cellulases of several fungi and molds, Reese studied the effect of substituents, esterified or etherified with cellulose (143). A substituent group on each glucose unit prevented enzyme action, but unsubstituted regions within chains were susceptible to hydrolysis. Some fungi have an enzyme which can remove acetyl groups from octaacetylcellobiose, but not from cellulose acetate. Such fungi can grow in a medium containing only octa-acetylcellobiose as a carbon source.

A partially purified cellulase preparation from the hepatopancreas of snails produced only glucose from cellulose. It did not form cellobiose and other oligosaccharides (144), but this result most probably arose from the presence of glycosidases. Cellulolytic enzymes in the extract from micro-organisms in sheep rumen were studied by Festenstein. The cellobiase was inhibited strongly by gluconolactone, whereas cellulase (CMCase) was less inhibited. CMCase appeared to produce a small amount of cellopentaose from carboxymethylcellulose (CMC) in addition to cellobiose (145).

By fractionation of *Myrothecium verrcaria* culture medium, multiple cellulolytic components were obtained, one capable of attacking only long chains and another relatively short chains (146).

One such fraction was studied by Thomas & Whitaker for its action on methyl β-cellobioside and higher members of the series; all were hydrolyzed (147). By cellulose column chromatography, Hash & King observed that several cellulolytic and β-glucosidase components were separated from the culture fluid of *M. verrcaria*. Of the cellulolytic components, one removed glucose units from insoluble cellodextrins, whereas the other split off only cellobiose (148). The former fraction seems to correspond to the cellulase

in the fraction investigated by Thomas & Whitaker (147). The latter appears to resemble the cellulase which was obtained by Nisizawa (149) in a crystalline form from *Irpex lacteus*. This cellulase produced mainly cellobiose from hydrocellulose as well as from *p*-nitrophenyl β-cellobioside. Hash & King found further that an aryl β-D-glucosidase from *Myrothecium* showed no activity toward cellobiose and transferred a β-glycosyl residue to various alcohols (150).

Sison, Schubert & Nord (151) tried to purify the cellulolytic components of *Poria vaillantii*. The purified product still showed two peaks in electrophoretic patterns. The faster moving was cellulase and the slower β-glucosidase. The cellulase was activated by KCN, $NaNO_3$, hydroquinone, and pyrocatechol and was inhibited by $CuSO_4$ mixed with $K_3Fe(CN)_6$. These results suggested the presence of an -SH group in the enzyme.

Using dichromate oxidation, Halliwell devised a micromethod for the determination of residual cellulose in the reaction mixture from cellulase action (152).

Celluloses are usually considered to be utilized by herbivores because of the digestive action of bacteria in intestines. Conrad *et al.* (153) also reported similar results for rats. Approximately 50 per cent of administered cellulose-C^{14} disappeared in the intestine as a result of the action of bacteria. A significant amount of C^{14} was also found in a petroleum ether-soluble material in the feces.

β-Glucosidases.—Various oligosaccharides such as laminaribiose, gentiobiose, sophorose, gentiotriose, and other unknown oligosaccharides were found by Crook & Stone after action of *Aspergillus niger* extract on cellobiose. These oligosaccharides were also formed when the enzyme extract acted on laminaribiose, gentiobiose, methyl β-D-glucoside, or salicin (154). When D-xylose was used as acceptor, 3-O-β-D-glucopyranosyl-D-*xylose*, laminaribiose and unknown disaccharides were obtained by the action of *A. niger* β-glucosidase on cellobiose (155).

Some bacterial β-glucosidases act as transglucosylases. From the culture medium of *Acetobacter xylinum* of a cellulose-forming strain growing on glucose, cellobiose and cellotriose were isolated, whereas only glucose was found in the culture medium of a noncellulose-forming strain (156). The presence of an enzyme which catalyzed the formation of cellobiose, a β-linked disaccharide, from α-D-glucose-1-phosphate and glucose was found in the culture medium of *Clostridium thermocellum* (157).

Almond emulsin enzymes showed a specificity for some N-alkyl derivatives of salicylamide β-D-glucosides somewhat different from that for the same derivatives of β-D-galactosides. The emulsin showed activity toward these β-D-glucosides in the order: H > methyl >>> *n*-propyl >> dimethyl = isopropyl. It showed the order toward the corresponding β-D-galactosides: H >> methyl >>> *n*-propyl ≃ dimethyl > isopropyl. On this basis, a difference between β-glucosidases and β-galactosidases was suggested by Wagner & Kühmstedt (158). Helferich & Jung (159) arrived at the same conclusion, since these β-glycosidases in almond emulsin were

adsorbed differently by poly-(p-hydroxystyrene β-ᴅ-glucoside) and poly-(p-hydroxystyrene β-ᴅ-galactoside).

Several o- and p-acylphenyl β-ᴅ-glucopyranosides such as those from acetophenone and propiophenone were synthesized by Wagner. Whereas the *para*-substituted products showed the same ease of hydrolysis by almond emulsin, the *ortho* isomers showed a decrease in rate of hydrolysis with an increase in chain length and in chain branching of the acyl groups (160). Somewhat different results were observed by Wagner with several o- and p-alkylphenyl β-ᴅ-glucosides of increasing chain length of alkyl groups. The activities of almond emulsin toward the *ortho* isomers decreased gradually with an increase in chain length except for the equal activities toward the n-propyl and n-butyl derivatives, whereas the ease of hydrolysis of the *para* isomers increased with an increase in chain length (161). For the salicylamides of β-ᴅ-xylopyranoside and α-ʟ-arabopyranoside, N-alkylation of the aglycon group also slowed the rate of splitting by almond emulsin (162).

The specificity of transglucosylases from several higher plants using p-nitrophenyl β-ᴅ-glucoside as substrate and several alcohols as acceptors was studied by Suzuki. The specificity differed according to origin (163).

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THE LIPIDES[1]

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Since research work in the field of lipides has recently increased greatly, this review will deal only with a few selected topics. Furthermore, the authors wish not to repeat any material already reviewed and therefore cite, at the beginning of the different sections of this chapter reviews which may be consulted for earlier work.

FATTY ACIDS

It is the purpose of this section to supplement certain of the topics of Shorland's review (1). The metabolism of fatty acids was recently reviewed by Lynen (2), by Kennedy (3) and by Green (4).

ANALYSIS AND SYNTHESIS

Normal fatty acids (C_{12} to C_{18}, odd- and even-numbered, and C_{20}, C_{22}, C_{24}, and C_{26} fatty acids) have been crystallized from different solvents, such as pentane, ethyl ether, alcohol, etc. The solid phases have been investigated from the polymorphic point of view by Sydow (5), using an x-ray powder method. The same author (6) found different infrared spectra of the different crystal forms of the same normal fatty acid. The parts of the spectra between 7.7μ and 8.5μ and near 11.0μ are the best for identification purposes. Linstead *et al.* (7) presented evidence for the *cis* configuration of natural eicos-11-enoic acid (which is the chief component of jojoba oil and also occurs in the glycerides of Atlantic cod-liver oil), after synthesizing the 11,12-dihydroxyarachidic acid obtained by anodic crossed coupling of a 9-10-dihydroxystearic acid with benzyl hydrogen succinate. Youngs, Epp, Craig & Sallans (8) have found a rapid method for preparing the long-chain fatty acid chlorides which eliminates purification by distillation. Working with oleic, stearic and tetrabromostearic acid, the authors obtained quantitative recovery of the product containing less than 1.5 per cent free acid. The preparation of a great variety of linoleic acid derivatives in which brom addition products of linoleic acid were used as starting materials was reported by Kaufmann & Stamm (9). Magne *et al.* (10) described a method for the purification of saturated fatty acids based upon the recrystallization of the fatty acid–acetamide molecular compounds and subsequent regeneration of the acid by extraction of the acetamide with water. It was shown by Hallgren, Stenhagen & Ryhage (11), using the mass spectrometric method, that the peak heights of the parent peaks can

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[1] The survey of the literature pertaining to this review was completed in August, 1958.

be used for the quantitative analysis of mixtures of normal-chain fatty acid esters of high molecular weight even for mixtures such as stearic, oleic, linoleic, and linolenic acid esters. However, in a complex mixture it is not possible to distinguish between geometrical and positional isomers. Perhaps it may be possible to overcome this difficulty by examining suitable derivatives. Studying the isomerization of polyunsaturated fatty acids, Sreenivasan & Brown (12) estimated the conjugation products after isomerization of linolenic acid in a sealed tube for 2 hrs. at 140° with potassium-t-butoxide in t-butanol. Under these conditions, conjugation of linolenic acid and arachidonic acid appears to have attained completion. Rudloff (13) determined the position of double bonds in unsaturated fatty acids and esters by a periodate-permanganate oxidation method. With oleic and other monoenoic acids the author obtained quantitative results in the aqueous reaction mixture. Linoleic acid reacted too fast at normal concentrations, and no malonic acid was isolated.

For studying specific problems of lipide metabolism, it appears to be desirable to use randomly labeled fatty acids. These were synthesized by *Chlorella pyrenoidosa,* growing in an atmosphere of CO_2 and $C^{14}O_2$, as reported by Mangold & Schlenk (14). A semimicro method for the separation and stepwise degradation, in which the carboxyl carbon of the fatty acid is removed and isolated as the carboxyl carbon of benzoic acid, was described by Gibble *et al.* (15).

Chromatographic separation.—By slightly modifying the method of reversed-phase partition chromatography used by Howard & Martin (16), Kapitel (17) got good results in separation of the even-numbered C_6 to C_{22} fatty acids and even fractionation of a mixture of oleic, linoleic and linolenic acid was reported. Another extension of this kind of chromatography was given by Wittenberg (18) for the C_6 to C_{12} fatty acids and by Garton & Lough (19), including odd- and even-numbered C_8 to C_{20} fatty acids. It is interesting that reversed-phase partition chromatography is also suitable for mixtures of acetylenic, ethylenic, and saturated acids encountered in the synthesis of *cis*-long-chain fatty acids by catalytic semihydrogenation, as is reported by Crombie (20). With the technique of carrier displacement chromatography (21, 22), the separation of some of the fatty acids of milk has been shown to be possible by Kuramoto *et al.* (23).

A short summary of gas-liquid chromatography has been given by James (24). Using the highly sensitive detector (gas-density meter) (25), James & Wheatley (26) determined the component fatty acids of human forearm sebum. With the method described by James & Martin (27) it has been shown that not only the series of acids ranging from n-heptanoic to n-octadecanoic acid, but also two series of branched-chain acids are present in human sebum (26). The highly branched acids should be restricted to the odd-numbered acids, while the simple methyl branches occur with both odd- and even-numbered acids. Both mono- and dienoic acids of a variety of

chain lengths are also present. After collecting different samples from the gas chromatogram, James & Webb (28) oxidized the unsaturated acids with $KMnO_4$; the mono- and dicarboxylic acids which were produced were identified on another chromatogram. Thus the structures of some naturally occurring unsaturated acids have been established. After oxidation of linolenic acid according to Lemieux & Rudloff (30), however, Nowakowska *et al.* (29) obtained a considerable number of unexpected products. A rapid gas chromatographic analysis of fatty acid methylesters up to C_{26} was described by Beerthuis & Keppler (31). For solving the problem of separation of unsaturated fatty acids such as linoleate and linolenate, etc., Lipsky & Landowne (32) proved adipate polyester of diethylene glycol to be an extremely efficient phase. Retention times for a number of saturated and unsaturated fatty acids under experimental conditions optimum for good resolution were reported by Orr & Callen (33). Very recently a robust but sensitive detector based on the measurement of the changes in dielectric constants was described by Turner (34).

Paper chromatography.—Since Shorland's review in 1956 (1) much work has been done on the paper chromatography of fatty acids. Although chromatography on impregnated filter paper (35, 36) is very useful for the separation of saturated fatty acid mixtures and also for mixtures of unsaturated acids with the same chain length (37, 38), there are two difficulties: first, to separate "critical pairs" (39), and, second, to find methods for quantitative chromatography. Schlenk *et al.* (40), using siliconized paper as stationary phase and different solvent mixtures as mobile phase (40, 41), increased the R_F values of the saturated and unsaturated acids by chromatographing at low temperatures. Combining the technique at $-30°$ in one direction and the chromatography at $+20°$ on the same paper in the other direction, Kaufman & Mohr (42) obtained good separation of a fatty acid mixture consisting of saturated and unsaturated acids with different chain lengths. Fries *et al.* (43) treated the chromatographed unsaturated fatty acids with ozone and sprayed them with fuchsin-sulfurous acid. Besides the large variety of different indicators reported earlier (1), Mangold *et al.* (44, 40) found α-dextrin and subsequent treatment with iodine vapor to be successful as an indicator on chromatograms not only for fatty acids, but also for their esters and for fatty alcohols. The area covered with lipide remains white, while the remainder of the paper turns bluish purple. Burness & King (45) have tested a method suitable for fatty acids up to C_{10}. The fatty acids are run in a mixture of three volume 3 N aqueous ethylamine and seven volume N butanol, and, after drying of the papers, the salts are sprayed with ninhydrin and collidine in ethanol.

Quantitative paper chromatography of fatty acids has been reported by Wagner, Abisch & Bernhard (46) and by Seher (47), who determined not only the density of the paper strip, indicating the acids as their copper salts, but also measuring the areas under the absorption curves. Perilä (48) pro-

ceeded similarly but worked with silver salts of the fatty acids. The quantitative use of mercuric acetate complexes with unsaturated acid methyl esters according to Inouye et al. (49) has been reported by Kaufmann (50) and by Schmidt (51). Very recently Ballance & Crombie (52) described the paper chromatography of 40 different fatty acids on a qualitative and a quantitative scale. It should be mentioned that the method of Kaufmann & Nitsch (36) can be applied to the separation of mono- and polyoxy acids and to di-, tetra-, and hexabromostearic acids (53). To settle the difficulties of separation of fatty acids with higher chain length than C_{20}, Kaufmann & Pollerberg (54) investigated the allyl ester compounds with good results. Schulte & Storp (55) described the photometric measurement of paper chromatograms of long-chain aldehydes and ketones. Aliphatic methylketones and aldehydes from C_1 to C_8 were separated and identified by Wallgren & Nordlund (56) as 2,4-dinitrophenylhydrazones on untreated paper, with 10 per cent acetic acid in heptane (ligroin) as the moving phase. Seher (57) paper chromatographed the C_2 to C_{10} dicarboxylic acids in the form of their ammonia salts in 78 per cent alcohol (v/v) and stained with ninhydrin.

POLYENOIC FATTY ACIDS OF ANIMAL ORIGIN

Structure.—Although many members of the polyunsaturated fatty acids are known to exist in lipides of animal origin differing by chain length and number and position of double bonds, few of these acids have been adequately characterized. The occurrence of polyunsaturated fatty acids of the C_{20} and C_{22} series in fish oils was shown by Bull (58). The best known of these acids was the clupanodonic acid, $C_{22}H_{34}O_2$, found by Tsujimoto (59). Another polyenoic acid occurring in mammalian liver phosphatides, eicosatetraenoic acid, named later arachidonic acid, was first isolated by Hartley (60). The structure of this arachidonic acid was investigated by Smedley-Maclean et al. (61, 62) and proved to be $\Delta^{5, 8, 11, 14}$-eicosatetraenoic acid. Although some other C_{20} and C_{22} polyunsaturated fatty acids had been isolated by different authors from fish oils (see 63 to 67), the supposed positions of double bonds were not uniform. They depended, rather, on the methods employed. Klenk & Bongard (68) described a method of oxidative ozonolysis which enabled the authors to get 50 per cent of the theoretically expected malonic acid as split product of the divinyl-methane pattern $(-CH=CH-CH_2-CH=CH-)$. Investigating the structure of the polyenoic acids, they estimated by chromatographic analysis the composition of the dicarboxylic acid mixtures derived after ozonolysis both from the esters and from the the free acids. Thus it was shown that the polyenoic acids of the glycerophosphatides of brain (69), liver (70) and also from fish-liver oils (71) possess the divinyl-methane pattern, because the only free acid after ozonolysis of the esters was malonic acid. The ultraviolet absorption curves after alkaline isomerization according to Holman & Burr (72) and the

occurrence of the different mono- and dicarboxylic acids after degradation indicated the presence of a great number of different polyenoic acids of the C_{20} and C_{22} series (1). Very recently Klenk & Brockerhoff (73) described

TABLE I

EVEN-NUMBERED n-POLYENOIC ACIDS OF HUMAN BRAIN PHOSPHATIDES (B), BEEF LIVER PHOSPHATIDES (L), AND FISH OILS (F) INVESTIGATED FROM 1955 TO 1958

Chain length	Position of the double bonds		Occurrence		
	Counted from the carboxylic group	Counted from the CH₃ group	B	L	F
C_{18}	9, 12*	6, 9		(70)	(73)
	9, 12, 15*	3, 6, 9		(70)	(73)
	6, 9, 12, 15*	3, 6, 9, 12			(83)
C_{20}	8, 11†	9, 12		(37)	
	11, 14‡	6, 9	(69, 79)	(37)	
	5, 8, 11†	9, 12, 15	(69, 79)	(70, 37, 81)§	
	8, 11, 14†	6, 9, 12	(79)	(81, 37)	
	5, 8, 11, 14*	6, 9, 12, 15	(69, 79)	(70, 37, 81)	
	5, 8, 11, 14, 17*	3, 6, 9, 12, 15		(70, 37, 81)	(82, 84, 87)
C_{22}	10, 13†	9, 12		(38)	
	7, 10, 13†	9, 12, 15	(69, 76)	(38)	
	7, 10, 13, 16*	6, 9, 12, 15	(69, 78)	(38)	
	4, 7, 10, 13, 16†	6, 9, 12, 15, 18	(69, 78, 76)		
	7, 10, 13, 16, 19*	3, 6, 9, 12, 15	(69, 76)	(70, 38)	(73)
	4, 7, 10, 13, 16, 19*	3, 6, 9, 12, 15, 18	(69, 78)	(70, 38)	(73, 85)
C_{24}	9, 12, 15, 18‡	6, 9, 12, 15	(80)		

* Isolated in a pure state.

† Isolated as mixture with other fatty acids of the same chain length.

‡ Isolated with small amounts of impurities.

§ This fatty acid has also been found by Mead & Slaton (86) from fat-deficient rat organs.

a method of reductive ozonolysis of the polyenoic acids. After reductive ozonolysis of the acids, the aldehydes which were formed were obtained by distillation and converted into the corresponding hydrazones. After separation on silicic acid columns according to Roberts & Green (74), the hydrazones were determined photometrically, isolated, and identified as well by

paper chromatography (75) as by determining the melting point. While the longer dicarboxylic acids obtained by oxidative ozonolysis indicate the position of the first double bond counting from the carboxylic group, the aldehydes which are obtained by reductive ozonolysis give the position of the first double bond counting from the CH_3 end of the polyenoic acids.

Both methods confirmed the theory that the investigated polyunsaturated C_{18}, C_{20}, and C_{22} fatty acids (see Table I) mainly belong to the linoleic or linolenic acid type (76). For further investigation it was of importance to isolate the different acids of the mixture. Using both the vacuum distillation and the countercurrent distribution techniques (77), Klenk & Lindlar isolated $\Delta^{7, 10, 13, 16}$-docosatetraenoic and $\Delta^{4, 7, 10, 13, 16, 19}$-docosahexaenoic acid (78) from the C_{22}-polyenoic acid fraction, and $\Delta^{5, 8, 11, 14}$-eicosatetraenoic acid (79) from the C_{20}-polyenoic acid fraction of brain. Further investigations of the highly unsaturated fatty acid mixtures of brain phosphatides (80, 76), beef liver phosphatides (37, 38, 81) and fish oils (73, 82, 83) resulted in the isolation of numerous kinds of polyenoic acids in Klenk's laboratory. Most of them were obtained in a pure state; they migrate as entities when chromatographed on paper (37, 38, 81). Some of them are contaminated with small amounts of closely related acids. But also in these cases the nature of the acids could be established by the results obtained after degradation. $\Delta^{5, 8, 11, 14, 17}$-eicosapenta- and $\Delta^{4, 7, 10, 13, 16, 19}$-docosahexaenoic acid have also been isolated by Whitcutt & Sutton (84) and Whitcutt (85) from fish oil. All the new-found fatty acids are shown in Table I. It should be mentioned that Klenk & Montag (80) found a polyenoic acid with a chain length of C_{24}. From the phosphatides of brain they isolated $\Delta^{9, 12, 15, 18}$-n-tetracosatetraenoic acid, which is present only in small quantities.

When the individual fatty acids of Table I are compared, it is obvious that they belong to three kinds of structure types, if one counts the double bonds beginning at the terminal CH_3 group. The first type possesses the first double bond in the same position as oleic acid and is therefore called the oleic acid type. The corresponding second and third types are the linoleic and the linolenic acid types respectively, because the first double bond is in the same position as that of linoleic or linolenic acid respectively (87). As is to be seen from Table I, the polyenoic acids of the linoleic acid type are present in brain and liver phosphatides, but they are almost absent from fish oils or are present only in traces, while the polyunsaturated acids of the linolenic acid type are predominant components of fish oils. A comparison of the composition of the C_{20} and C_{22} fatty acid mixtures of different origin confirms this.

Biosynthesis.—The importance of certain fatty acids was demonstrated by the work of Burr & Burr (88). Their work and that of subsequent investigators showed that the rigid exclusion of fat from the diet of rats induced cessation of growth, scaliness of skin, kidney damage and impaired

reproduction. All these abnormalities can be prevented or cured by oral supplements of small amounts of linoleic or arachidonic acid. Linolenic acid has been shown to stimulate the growth of fat-deficient animals, but it is unable to cure the dermal symptoms of the deficiency (89, 90, 91). In addition, Bernhard & Schoenheimer (92) and Bernhard *et al.* (93) showed, that the linoleic acid occurring in animal organs was not synthesized but taken up with food by the animals. So the findings of Burr & Burr were gradually accepted, and the term "essential" was eventually used for all polyunsaturated fatty acids, although this term should include only those substances which are active both for growth and for maintenance of dermal integrity (94). Thomasson's work (95) has suggested that the presence of double bonds at the 6 to 7, 9 to 10 positions counting from the terminal CH_3 group is fundamental for the biological activity. While Hume *et al.* (96), assaying a C_{22} fraction obtained from cod-liver oil, found on test animals only a slight positive reaction. De Jongh & Thomasson (97) tested a C_{22} polyunsaturated fatty acid fraction of brain (containing a mixture of polyenoic acids of linoleic and linolenic acid type) which gave evidence for a significant potency of this fraction.

Nunn & Smedley-MacLean (98) reported that the livers of fat-deficient rats contained no arachidonic or higher unsaturated fatty acids. Supplementing the diet with linoleic acid resulted in the production of arachidonate. After having determined the structure of arachidonic acid (61, 62), Smedley-MacLean (96) presumed that this acid is synthesized in liver from linoleic acid by extension of the chain, for two C atoms, *i.e.*, by addition of acetate with simultaneous or subsequent dehydrogenation. The occurrence of $\Delta^{5, 8, 11, 14}$-eicosatetraenoic (79), $\Delta^{7, 10, 13, 16}$-docosatetraenoic (78), and $\Delta^{9, 12, 15, 18}$-tetracosatetraenoic (80) acid in the phospholipides of brain is an example of the simple chain extension. Chain extension with simultaneous dehydrogenation which introduces the new double bond in the divinyl-methane pattern toward the carboxylic group, as discussed by Klenk (99), is to be seen in comparing the $\Delta^{6, 9, 12, 15}$-octadecatetraenoic (83) and $\Delta^{5, 8, 11, 14, 17}$-eicosapentaenoic (82, 84, 87) and the $\Delta^{4, 7, 10, 13, 16, 19}$-docosahexaenoic acid respectively (73, 85), which occur in especially large amounts in fish oils.

Proving the theory on the biosynthesis of the polyenoic acids in mammalian organisms, at the same time Mead *et al.* (100) and Klenk (101) studied the incorporation of carboxyl-labeled acetate into the polyenoic acids. Mead *et al.* (100), confirming the findings of Bernhard & Schoenheimer (92), showed that acetic acid is not incorporated into linoleic acid of the lipides of weanling rats. Arachidonic acid, on the other hand, was derived from acetate and, presumably, an exogenous C_{18} precursor. The C^{14} of acetate was found by Klenk (102) to be mainly in succinic and glutaric acids among the dicarboxylic acids formed by degradation of the polyenoic acid mixture after administration in experiments with rats and rat liver

slices. C^{14} was also found in the higher dicarboxylic acids, while malonic acid contained only very small amounts. Further work on the metabolism of essential fatty acids gave no evidence for the conversion of oleate to linoleate (103), and the distribution of the label of arachidonic acid isolated from rats after feeding experiments showed again that this acid was synthesized in the rat by condensation of linoleate with acetate (104).

Brain tissue was shown to be able to incorporate C^{14} acetate into polyenoic acids. Klenk (105), when using tissue slices, obtained much higher uptake of labeled acetate than is obtained in feeding experiments. It is interesting that under the same conditions no uptake of C^{14} could be found in other lipide elements, such as cerebronic acid, cholesterol, sphingosine, and aldehydes. Smedley-MacLean (98) had already shown that the livers of rats fed on a fat-free diet were nearly free of arachidonic acid, while a C_{20}-trienoic acid was accumulated. These findings were confirmed by Mead & Slaton (86) and also by Dam & Engel (106). According to Mead & Slaton, this acid is the $\Delta^{5,8,11}$-eicosatrienoic acid found by Klenk et al. (37, 70, 81) to be present in small amounts in liver phosphatides of normal animals. While Mead & Slaton (86) supposed that this trienoic acid would be a partial hydrogenation product of arachidonic acid rather than an intermediate in the conversion of linoleic to arachidonic acid, Dam & Engel (106) discussed the possibility that the trienoic acid would be formed from oleic acid or even from saturated acids in fat deficiency. Consequently, the organism would be able to synthesize the polyenoic acids to a special degree. However, only those of the oleic acid type would be built. This supposition agrees with the experimental results by Klenk (101, 105) when investigating the uptake of C^{14}-acetate in the polyenoic acids. The malonic acid obtained after ozonolysis was only slightly, but quite noticeably radioactive.

In chicks also, an essential fatty acid deficiency produced a marked decrease in dienoic and tetraenoic acids and an increase in trienoic acids, as described by Bieri et al. (107). In rats fed fat-free diet supplemented with methyllinolenate, Mead (108) found no linolenic acid, but he did find a docosapentaenoic acid and also an increasing of palmitoleic acid. Some years before, similar results were obtained by Rieckehoff, Holman & Burr, feeding corn oil or cod-liver oil (109). Pyridoxine was shown by Witten & Holman (91) to stimulate polyenoic acid synthesis in fat-deficient rats supplemented with linoleate or linolenate. When methyl-γ-linolenate-1-C^{14}-$\Delta^{6,9,12}$-octadecatrienoic acid was fed to rats, the sole highly active fatty acid was arachidonic acid isolated by Mead & Howton (110). These results seem to support the hypothesis that γ-linolenic acid is an intermediate in the conversion of linoleic acid to arachidonic acid. As was shown by Whitcutt (85), the $\Delta^{4,7,10,13,16,19}$-docosahexaenoic acid received from South African pilchard oil possesses the all cis configuration. Estimating the essential fatty acid activity of ethyl cis-9,trans-12-linoleate, Privett et al. (111) found it

to possess nonpotency. *Trans* double bonds derived from this acid are retained by the animal and are deposited in tissue lipides. Although some work was done, we do not know very much about the metabolic importance of these "essential" fatty acids. That they do not depress tuberculosis *in vitro* was shown by Weitzel (112).

PHOSPHOLIPIDES

For the chemistry of the phosphatides the review by Baer (113) and Lovern's book (114) may be consulted; the metabolism of phosphatides is treated in the reviews of Zilversmit (115), of Kennedy (3) and of Dawson (116).

ANALYTIC METHODS

A sensitive and simple method for the estimation of free amino groups in unhydrolyzed phospholipides was given by Lea & Rhodes (117). The method is based on that of Moore & Stein (118) for amino acids, using ninhydrin in buffered methylcellosolve. Since the method is rapid to use it has considerable value in connection with chromatographic and other separation methods of phospholipides from natural sources. On the basis of Lea & Rhodes's results, it appears that the error involved in calculating mixed amino-containing phospholipides on the basis of a color yield of 90 per cent diketohydrindylidene-diketohydrindamine would not be very great. Another determination of amino nitrogen in unhydrolyzed phospholipides, proposed by Wheeldon & Collins (119), depends on the quantitative preparation of dinitrophenyl lipides and the subsequent measurement of their light absorption. 1-Fluoro-2,4-dinitrobenzene was shown by the authors to react in the presence of triethylamine with the free amino groups of the phospholipides dissolved in benzene. A specific method for the estimation of the higher fatty aldehydes of tissue lipides converting these into their *p*-nitrophenylhydrazones and measuring these colorimetrically is presented by Wittenberg *et al.* (120). The advantage of this method is that the lower aldehydes do not interfere under the conditions described. By combining the methods of Appleton *et al.* (121) and of Kushner (122), a new modification of the periodide method for choline determination was obtained by Smits (123). The choline periodide precipitate formed by addition of KI_3 reagent to a choline solution, is taken up in iodine containing ethylene dichloride in the presence of water. The interfering I_3 ions remain in the water phase and the extinction of the ethylenedichloride layer resulting from choline triiodide is measured. Besides the useful yeast bioassay method for inositol of Taylor & McKibbin (124), Böhm & Richarz (125) described a chemical one, which is based on the oxidation with periodic acid. The lipide sample is first hydrolyzed, the hydrolysate is separated by paper chromatography, and the inositol spots eluated.

EXTRACTION METHODS

Folch and co-workers have developed a very useful method for the preparation and purification of brain lipides (126). A recent paper describes a simplified version of this method (127) and reports the results of a study of its application to different tissues such as the gray and white matter of brain, liver and muscle. The method consists of homogenizing the tissue with a 2,1-chloroform-methanol mixture and washing the extract by addition to it of 0.2 volume of either water or an appropriate salt solution. The washing procedure removes essentially all the nonlipide contaminants with a concomitant loss of less than 1 per cent of the tissue lipides. Methods are described by Kates & Eberhardt (128) for the extraction of total lipides from leaves under conditions minimizing the action of phosphatidase-C, which is greatly accelerated by use of ethyl ether or alcohol ether mixtures.

CHROMATOGRAPHIC METHODS

Preparative chromatography.—Chromatography of phospholipides of egg on alumina has been employed by Rhodes & Lea (129). With methanol-$CHCl_3$ (1:1 v/v) and ethanol-$CHCl_3$-water (5:2:2 by volume) the authors obtain a sharp separation into choline-containing and noncholine-containing fractions with quantitative recovery of both, in contrast to the older chromatographic technique of Hanahan *et al.* (130), which was a purification method of lecithin. It is necessary, however, to separate the lyso compounds from the "lecithin" or "cephalin" fractions by the use of chromatography on silicic acid (129, 131, 132). Within the past few years, the availability of pure silicic acid has made possible its application to the chromatographic separation of the phospholipides. Thus chromatography on silicic acid columns has been investigated by different authors (128, 133, 134, 135), but it is difficult to obtain pure identical lipide fractions and reproducible results. The adsorbing power of silicic acid varies among the different preparations and sometimes it is necessary to activate the silicic acid by drying at 105° (135) or prewashing with methanol-chloroform (134). Garton & Duncan (136) also activated silicic acid before chromatographing blood lipides of the lactating cow and obtained a good separation of the cholesterol esters, triglycerides, sterols and phospholipides. While Marinetti *et al.* (134) investigated pig heart phospholipides, Hanahan *et al.* (135) obtained phospholipide fractions of rat liver, beef liver, and yeast in a reasonable state of purity. Of considerable interest was the finding that 90 to 95 per cent of the inositol containing phospholipides could be obtained in a single fraction, while McKibbin (133) using silicic acid chromatography observed, that inositides are present in significant amounts in several different fractions. The gradient elution technique was intended by Brolin (137) to concentrate the lipides from eye lense into definite fractions but these chromatograms did not give a complete separation.

Paper chromatography.—Paper chromatography in the phospholipide

field can be applied to (a) the lipide constituents, e.g., hydrolysis products, and (b) the total lipide extracts. The technique used to separate the hydrolysis products does not appear to be very difficult. If the chromatograms are developed under identical conditions, the R_F values differ only in a small range (138). Measuring the areas with a planimeter after development, Olley (139) reported a quantitative method for a few specific constituents of lipide hydrolysate.

The major work on the paper chromatography of the phosphatides on nonimpregnated filter paper occurred in the period preceding 1955. The solvent systems were only partially successful. Solvents consisting of mixtures of ketones and acetic acid gave a greater degree of separation of lecithin and phosphatidylethanolamine (140), although it should be remarked that the plasmalogens can be decomposed with acetic acid. A special method to separate "free" plasmal besides the plasmalogen was given by Thiele (141). Douste-Blazy, Polonovsky & Valdiguié (142) proposed a separation method in which partition occurs between two nonmiscible organic phases, a nonpolar solvent (ascending) and a polar solvent mixture (descending). R_F values are calculated from the meeting point. By this method it was possible to separate and identify some blood lipides (143). After using silica-impregnated paper for phospholipide chromatography by Lea & Rhodes (132, 144) with the solvent system of methanol-chloroform (methanol 20 to 30 per cent in chloroform v/v), Marinetti et al. (145) obtained good separations with solvent mixtures of diisobutylketone acetic acid, and water. The latter authors cut the spots off, eluted the phosphatides from the developed chromatograms and estimated the P after digestion. The same method was used on a qualitative scale to study the incorporation of P^{32}-labeled orthophosphate into the individual phosphatides of various tissues of the rat (146). A reasonably reproducible method has been developed by Dieckert & Reiser (147) for separating lysolecithin, lecithin, sphingomyeline and phosphatidylethanolamine, using glass-fiber filter paper impregnated with silicic acid as chromatographic medium and 1,1 methanol-ethyl ether as the developing solvent. A very quick separation of a lecithin- and lysolecithin-containing mixture was described by Rikimaru (148), who used untreated or siliconized filter-paper disks with chloroform-methanol (1:1) as developing solvent. Since the separation of the lipide mixtures depended on the degree of paper impregnation, it was always rather difficult to get reproducible results. Beiss & Armbruster (149) investigated other solvent mixtures, e.g., tetrahydrofuran-diisobutylketone-water (45:5:6) on untreated paper. Their separations of crude lipide extracts in one- and two-dimensional chromatograms are effective, and the method is quite simple to carry out.

SYNTHESIS

For the synthesis of phospholipides the reviews by Baer (113, 150) may be consulted. Another review by Malkin & Bevan (151) is worthy of men-

tion. Finally, Malkin (152, 153) gave two short introductions into the synthesis of phospholipides.

A procedure which permits the synthesis of the optically pure enantiomeric forms of unsaturated α-lecithins has been developed by Baer *et al.* (154). The starting material (D-acetoneglycerol) was phosphorylated by phenylphosphoryl dichloride, and the resulting product was esterified with ethylene chlorohydrin. The acetone-L-α-glyceryl phenylphosphoryl ethylene chlorohydrin thus formed was freed of its phenyl and acetone group and the L-α-glycerylphosphoryl chlorohydrin has been isolated in form of its barium salt. Treating this with oleyl chloride and pyridine in anhydrous dimethylformamide gave the corresponding dioleyl compound, which on heating with trimethylamine yielded a mixture of L-α-(dioleyl)-lecithin and oleyllysolecithin. The lecithin was readily soluble in 90 per cent acetone, indicating that the conventional procedures for the isolation of phosphatides from tissues permit loss of considerable amounts of the more unsaturated phosphatides (155). Tattrie & McArthur (156) prepared L-α-lecithin (dipalmitoyl) from L-α-glycerylphosphoryl choline by acylation with palmitoyl chloride in anhydrous chloroform in a yield of about 31 per cent. To prevent the formation of ethanolamine saltlike linkages in synthesized cephalins, Hirt & Berchtold (157) developed a new synthesis of cephalins (dipalmitoyl) using 2-hydroxyethylphthalimide phosphoric acid dichloride (phthaliminoethylphosphoric acid dichloride) as nitrogenous donator. The synthesis of cephalin described earlier (158) has now been extended to a new synthesis of phosphatidylserine by Bevan, Malkin & Tiplady (159). It has been prepared in good yield by the interaction of glycerol-1-iodide-2,3-distearate and N-benzyloxycarbonyl-DL-serine-benzyl ester-3-(silver phenyl phosphate) in boiling xylene in the dark, followed by catalytic hydrogenolysis. A method for the preparation of serine and 2-aminoethylphosphate esters is given in the same paper (159). A fully unsaturated cephalin (L-α-dioleyl cephalin) was prepared by Baer (160). Phosphorylation of α-β-diolein with phosphorus oxychloride in pyridine yielded the phosphatidyldichloride which on esterification with 2-hydroxyethylphtalimide gave phtaloyl cephalin. On removal of the phthaloyl residue a product was obtained which, after purification on a silica column, proved to be pure L-α-(dioleyl)-cephalin. An explanation of the "diazomethanolysis" reaction of Baer & Maurukas (161) was given by Brown & Osborne (162). The stability of some derivatives of 2-aminoethyl phosphate explains that the diazomethanolysis does not take place with lecithins, but only with phospholipides which possess amino groups.

A synthetic phosphatidyl peptide possessing a known structure should prove useful as a compound of reference in studies of the structure of natural lipopeptides and lipoproteins. Baer *et al.* (163) could accomplish the synthesis of O-(distearoyl-L-α-glycerylphosphoryl)-L-serylglycylglycine. Finally, *bis*(L-α-glyceryl) phosphoric acid has been prepared by Baer &

Buchnea (164). The procedure permits the preparation of all four isomers
(165). Thus it is possible to have reference substances for the hydrolysis
products of the recently obtained (166, 167) phosphatidyl glycerols which
represent a new class of phospholipides bearing a strong resemblance to the
phosphatidylinositols.

<h2 style="text-align:center">NATURALLY OCCURRING PHOSPHATIDES</h2>

Inositolphosphatides.—Phospholipides containing inositol (phosphoinosi-
tides) are known to occur in a great variety of plant and animal tissues
such as soybean (see 168 to 171), wheat germ (172), green peas (173),
groundnut (174), yeast (175), ox brain (176, 177), ox heart (178), egg
yolk (179, 180) and liver of horse and dog (133), of rat (135) and beef
(175). Evidence for the presence of inositol phosphatides in leaves was
given for the first time by Kates & Eberhardt (128). It is similar to that
found in soybean in that it contains sugars, although it has a much lower
P content. The inositol-containing phospholipides of brain seem to be di-
phosphoinositides with a complex structure based on inositol diphosphate,
as was shown by Folch (181). Inositol phosphatides of wheat germ (172),
heart (178), and liver (133) are known to have a structure very similar to
that of lecithin and cephalin, the molar ratios of fatty acid-glycerol-P-inosi-
tol being $2:1:1:1$. The possibility that McKibbin's monophosphoinositide of
liver (133) is a preparation artifact arising from hydrolysis during silicic
acid chromatography seems unlikely, since many fractions have been re-
peatedly chromatographed without any change. Recent work of Dawson
(182) has shown that monophosphoinositide of liver can be rapidly attacked
by phospholipase-B prepared from *Penicillium notatum*. From the digest a
water-soluble phosphatediester was isolated by paper chromatography con-
taining glycerol and inositol in equimolar proportions. Two fatty acids for
every water-soluble P atom were liberated. Free inositol monophosphate
has been isolated from liver (183). It is present in ox brain, ox heart and
in liver of various animals (183). Working with phosphoprotein prepared
from fresh rabbit liver by a method based on that of Phillips (184), Haw-
thorne (185) detected inositol and glycerol in equimolar quantities, proba-
bly derived from monophosphoinositide linked to the protein by saltlike
linkage. The work of Hutchison *et al.* (186) supports this conclusion, though
these authors do not specifically claim that the inositol they found arises
from phospholipide. Recent work of Brown & Higson (187) on hydrolysis
of esters of cyclohexanediol phosphates indicates phosphoryl migration also
during hydrolysis of the myoinositol-containing phospholipides to inositol
mono- or diphosphate. Further studies by Brown *et al.* (188) showed that
hydrolysis by acid or alkali of glycerol 1-(*cis*-2-hydroxy cyclohexyl phos-
phate) yielded mainly 2-hydroxycyclohexyl phosphate (about 85 per cent),
whereas the *trans*-isomer gave mainly glycerol phosphate (about 75 per
cent). Therefore, the predominant direction of breakdown after hydrolysis

of a phospholipide based on glycerol inositol phosphate could perhaps give information about the position of linkage of the glycerol phosphate residue to the myoinositol ring. Recent work of Hanahan & Olley (175) on the chemical nature of highly purified monophosphoinositides of rat liver, beef liver, and yeast leads to the conclusion that the most probable structure is that of a diacyl derivative of glycerylphosphorylinositol.

Cardiolipin and polyglycerolphosphatides.—The complex phosphatidic acid was considered by Pangborn (189) to be a glycerylglycerophosphate derivative containing three phosphoric acid molecules linking four glycerol molecules, the latter being also esterified with five linoleic and one oleic acid residues. Macfarlane & Gray (190) obtained cardiolipins from ox heart muscle by different methods and found a molar ratio P-glycerol-fatty acids of $1:1.5:2$. They threw doubt on Pangborn's formula, because of their results after alkaline hydrolysis and oxidation. The authors supposed the following structure: diglyceride-phosphoric acid-glycerol-phosphoric acid-diglyceride. Dawson (191) reported observations on a phospholipide fraction of rat liver, which enabled phospholipase-B of *P. notatum* to attack intact lecithin molecules. One of these two active lipides was shown to be a polyglycerol phospholipide very similar to that isolated by McKibbin & Taylor (192) from dog liver.

New and unidentified lipides.—Carter *et al.* (193) isolated a new ethanolamine-containing phospholipide from egg yolk which was present in the crude sphingolipide fraction. The purified lipide has been shown to consist of a phosphorylethanolamine derivative of batyl alcohol. The corresponding phosphatidic acid was first obtained by Klenk & Debuch (194), after reduction and hydrolysis of ethanolamine plasmalogen of brain. From the analytical data of the new phospholipide, it is concluded by Carter *et al.* (193) that the glycerol-ether has the alpha configuration, although this point will be confirmed later by the authors. Fractionating a benzene extract of bleached wheat flour by Craig distribution, Carter *et al.* (195) could isolate a lipocarbohydrate fraction, which gave 56 to 58 per cent of fatty acids after alkaline hydrolysis. The water-soluble products of both components yielded two distinct carbohydrate fractions, nonreducing before acid hydrolysis. After crystallization one of the compounds was identified as β-D-Galactosyl-1-glycerol, the other as α-D-galactopyranosyl-1,6-β-D-galactopyranosyl-1-glycerol. Another new lipide, phytoglycolipide, was found by Carter *et al.* (196, 197), which has the structural features of a glycolipide and of a phosphatide. Phytoglycolipide gives on hydrolysis phytosphingosine, fatty acids, inositol, glucosamine, a hexuronic acid, galactose, arabinose, mannose, and phosphate. The probable presence of more than one unidentified phospholipide in cod flesh was reported by Lovern *et al.* (198). For one of these they found a fatty acid-glycerol-P ratio of approximately $4:2:1$. In further study of the phosphate esters in the lipides of haddock and cod flesh after

hydrolysis, Olley (166) found different kinds of unknown phosphate esters, one, for example, which seemed to belong to the bisphosphatidic acid type. Another phosphate ester hydrolyzed at pH 4 only after preliminary hydrolysis with N HCl, giving one mole of glycerol to one mole of phosphorus. This is a known fact for substituted derivatives, e.g., methylglycerophosphate (199) and glycerylphosphorylcholine. Since the lipide had been hydrolyzed first with 0.5 N ethanolic KOH, this ester could not be glycerylphosphorylcholine, owing to the latter's rapid hydrolysis with ethanolic KOH. Similarly, the presence of the corresponding ethanolamine and serine esters was unlikely. Finally Nielsen (200), using the countercurrent technique of Craig (201), isolated lysophosphatidic acids and phosphatidic acids as components of the nonhydratable soybean phosphatides. Since these phosphatides are obtained after treatment with steam or hot water, it would be desirable to confirm these findings under milder conditions. Investigating the nitrogenous constituents of purified lipide extracts from various animal tissues after hydrolysis, McKibbin (202) found that the total N of brain, lung, and blood plasma is virtually accounted for by the known bases, whereas intestine, kidney, liver, and heart contain substantial amounts of undetermined N. Similar findings were reported by Olley (203). A beef liver serine and ethanolamine phospholipide fraction was found on the basis of a hydrolysate to contain 50 per cent of a nitrogen containing substance X, resistant to periodate (203). It stained with ninhydrin on paper chromatograms and, from the amount of glycerophosphate present in the hydrolysate, substance X seems to be a nitrogenous compound of a glycerophosphatide. It must be noted, however, that the liver phospholipide had been stored in $CHCl_3$ for four months at $-25°$ before hydrolysis. Investigating the composition of the phospholipides of cow's milk, Rhodes & Lea (204) could not identify some nitrogen, which was associated with the cephalin fraction, equivalent to 14 moles per cent of the lipide P. Finally, Collins & Wheeldon (205), preparing the dinitrophenylcephalines, reported of hitherto unrecognized forms of cephalin.

Plasmalogens.—Only once (206) has a method been reported for separating plasmalogens from the other glycerophosphatides. The method has never been published in detail. It seems to be extremely difficult to isolate pure native plasmalogens, since there is no method available. All our knowledge of the plasmalogens is based on results obtained in work with special "fractions" of glycerophospholipides consisting of lecithin and choline plasmalogen or of cephalin and the corresponding plasmalogen. The work on plasmalogens has been reviewed by Shorland (1) and Kennedy (3). In addition, Klenk & Debuch (194) in 1954 discussed three possible formulae for the natural occurring plasmalogens containing a fatty acid as well as a fatty aldehyde in the molecule. One of these (I) showed the aldehyde in the enol form, giving in effect an ether bond with glycerol:

$$CH_2-O-CH=CH-R$$

$$CH-O-\overset{\overset{\displaystyle O}{\displaystyle \|}}{C}-R$$

$$CH_2-O\diagdown\underset{\underset{\displaystyle OH}{|}}{\overset{\overset{\displaystyle O}{\displaystyle \|}}{P}}-O-CH_2CH_2NH_2$$

I
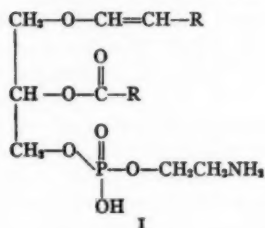
Two years later Rapport *et al.* (207) gave the same formula with respect to the linkage of the aldehyde residue. They treated a mixture of ethanolamine plasmalogen and phosphatidyl ethanolamine from bovine muscle with alkali and observed that the isolated acetalphospholipide, which had lost the fatty acid by this procedure, had an uptake of 0.88 mole of bromine per gram atom phosphorus. They concluded, therefore, that there must be a double bond in the molecule and that the aldehyde must be in the enol form. The plasmalogens of brain have more than 50 per cent of their aldehyde content as unsaturated aldehydes (208, 209). Possibly in the plasmalogen investigated by Rapport *et al.* unsaturated aldehydes have also been present. In a more recent work Rapport & Franzl (210) showed that the double bond of the enol ether linkage has a special uptake of iodine in methanol according to Siggia & Edsberg (211), while a usual olefinic unsaturated substance such as oleic acid did not react with iodine under these conditions. The fact that unsaturated ethers like vinyl-2-ethyl hexyl ether, vinyl isobutylether, and dihydropyran react with iodine to the same extent as do the plasmalogens confirmed the authors' earlier results. Independently, Debuch (212) investigated the nature of the linkage of the aldehyde residue. A phosphatidyl ethanolamine fraction of human brain prepared according to the chloroform-ethanol method of Folch (176) and purified by countercurrent distribution (213) was ozonized and split by further oxidation, yielding carboxylic derivatives. From these, longer chain monocarboxylic acids were separated. Two acids were recovered as main constituents, namely *n*-pentadecanoic and *n*-heptadecanoic acid, which represented the split product of an enol ether of hexadecanal or octadecanal. They were isolated and identified first with chromatographic methods (214) and more recently preparatively after distillation (215). Of course, all double bonds of the unsaturated aldehydes and acids were also oxidized. But these mono carboxylic acids had chain lengths of C_9 or less. Finally Blietz (216) confirmed the enol ether structure of the aldehyde in ethanolamine plasmalogen. He obtained a labeled plasmal (aldehyde) by cleavage of native plasmalogen in tritium water. The question whether the aldehyde is in the alpha or beta position has never been investigated by Klenk & Debuch. Rapport & Franzl (217) studied the action of snake venom lecithinase-A on preparations of beef

heart lecithin [prepared according to Pangborn (218)] containing 60 per cent plasmalogen. The authors found it to be hydrolyzed by the enzyme. The precipitate formed in the ethereal reaction mixture according to the method of Hanahan *et al.* (219) gave the fuchsin-sulfurous acid color reaction much more intensively than the ether solution. Since we know from the excellent work of Hanahan (220) that lecithinase-A from snake venom attacks the esterified primary alcoholic group of glycerol in lecithin, Rapport & Franzl concluded that the fatty acid ester group of the plasmalogen is formed with the primary and the aldehyde must therefore be linked through the secondary hydroxyl group of glycerol. Gray (221), investigating the position of the aldehyde residue in natural plasmalogens, found methyl glyoxal and phosphoryl choline or phosphoryl ethanolamine respectively, after oxidation and hydrolysis of lyso compounds obtained by splitting off the aldehydes from the choline or ethanolamine plasmalogens of ox heart muscle with acetic acid at 37°. This finding is in agreement with the results of Rapport *et al.* (217). Other authors reported of findings apparently at variance. Ansell & Norman (222) obtained after alkaline hydrolysis and subsequent mild acid treatment of rat brain ethanolamine plasmalogen only 59 per cent of the total phosphorus as α-glyceryl phosphoryl ethanolamine. If the aldehyde should be in the β position, no cyclic orthoester could be formed during hydrolysis with alkali; hence no β-glyceryl phosphoryl ethanolamine would be found. Marinetti & Erbland (223) reduced a purified pig heart plasmalogen fraction with hydrogen, and after alkaline hydrolysis they isolated a glycerol phosphate ether just as Klenk & Debuch isolated in the same manner from ethanolamine plasmalogen of brain (194) and from choline plasmalogen of beef heart (224). After hydrolysis with 6 N aqueous H_2SO_4 for 24 hr., Marinetti & Erbland (223) obtained 35 per cent of the corresponding ether which consumed on a molar basis one mole periodic acid with the concomitant liberation of one mole of long-chain aldehyde. The authors got the same results with the cephalin fraction of pig heart phosphatides and concluded, therefore, that the aldehyde of the natural plasmalogen should be in the alpha position of the glycerol. In extension of this work, Marinetti *et al.* (225) found the same melting point of the glycerol ethers and a synthetic D-α-octadecyl glycerol ether; the infrared spectra of these two compounds were identical. But they also found a β-glycerol ether, in another eluate after chromatography on silicic acid. So the obtained glycerol ethers of pig heart were mainly alpha derivatives (75 per cent alpha), but the beef heart glycerol ethers were predominantly beta (87 per cent beta). The authors explained the different results by a species difference. With respect to the fatty acids of the plasmalogen, Debuch (213) could not find any saturated ones in the ethanolamine cephalin fraction of brain and Klenk & Krickau (226) found only 4 per cent of the total fatty acids as saturated acids. Recent findings by Lovern *et al.* (227) are very interesting, in that these authors

found nearly 60 per cent of the lipides of ram spermatozoa as choline plasmalogens, although there was no evidence for the presence of lecithin.

SPHINGOLIPIDES

Components

In this group all lipides containing sphingosine or one of its derivatives [dihydrosphingosine (228), phytosphingosine (229), and dehydrophytosphingosine (230)] are included, i.e., sphingomyeline, cerebrosides and gangliosides or related substances. It is intended that this article should present only the results of recent work; the reader can find further detail in the reviews by Celmer & Carter (231), Baer (113) and Carter *et al.* (232).

Sphingosine (1,3-dihydroxy-2-amino-octadecene-4) and related compounds.—After the structure of sphingosine had been settled in general by the work of Klenk *et al.* (233, 234, 241) and Carter *et al.* (235, 236), the stereochemical questions could also be clarified. The infrared studies by Mislow (237) and Marinetti & Stotz (238) indicated a *trans* olefinic bond. Moreover, since the results of several laboratories (239, 240, 241) agree with respect of the configuration at the C atom 2, the D configuration seems to be proved. After developing an improved procedure for the preparation of psychosine from phrenosine, Carter & Fujino (242) obtained, by reduction of psychosine, dihydropsychosine, which on hydrolysis with ethanolic hydrochloric acid gave an excellent yield of erythro-dihydrosphingosine. No threo isomer could be detected in the hydrolysis product. Therefore it now seems to be certain that natural sphingosine has erythro configuration (243). Very recently the synthesis of erythro-*trans*-1,3-dihydroxy-2-amino-4-octadecene, racemic sphingosine and of its threo-*trans* (244), erythro-*cis* and threo-*cis* forms (245) was described by Grob & Gadient (246). The common precursors were 2-hexadecyne-1-al and nitroethanol, condensation of which yielded equal amounts of the erythro and threo forms of 1,3-dihydroxy-2-nitro-4-octadecyne. Since the latter can be converted to intermediates of erythro configuration, the synthesis of sphingosine is practically stereospecific. It should be pointed out that the availability of synthetic sphingosine and its stereo isomers is likely to have important consequences. The total synthesis of cerebrosides and sphingomyelines has come into much better view, and studies of the function and metabolism of sphingosine will certainly be facilitated. A mixture of racemic forms of phytosphingosine has been synthesized by Proštenik & Stanaček (247, 248). Finally, it should be mentioned that the same authors (249) drew from their results the conclusion that the sphingosine related base of yeast cerebrin consists of 2-amino-1,3,4-trihydroxy-*n*-eicosane. Brady & Koval (250) reported the preparation of an enzyme system obtained from rat brain tissue which catalyzes the incorporation of serine-C^{14} into sphingosine. It is apparent from their results, that the radioactivity from the serine-C^{14} is preferentially localized in positions 1 and 2 of sphingosine. In order to pursue studies of

sphingolipide metabolism, it is necessary to prepare derivatives of sphingosine to be used as substrates. Weiss (251) succeeded in synthesizing several phosphorylated derivatives of dihydrosphingosine, although the synthesis of sphingosine-1-phosphate was not achieved. N-Stearyl-and N-palmitylsphingosines were isolated for the first time by Marinetti & Stotz (252) from ox spleen. The identification of these amides was made on the basis of elementary analysis and a study of the products of hydrolysis of chromatography and infrared spectroscopy. A new colorimetric determination method of sphingosine base in lipides was given by Sakagami (253). The principle is based on the colorimetric determination of higher fatty aldehyde, produced by the oxidation of sphingosine base with lead tetraacetate.

Neuraminic acid.—Today there can be little doubt that neuraminic acid is the basic component recently described as sialic acid by Blix *et al.* (254, 294) and Werner *et al.* (255), as gynaminic acid by Zilliken, Braun & György (256), as lactaminic acid by Kuhn & Brossmer (257, 258, 259), and as hemataminic acid by Yamakawa & Suzuki (260, 261). It was recently proposed by Blix, Gottschalk & Klenk (262) that the unsubstituted compound be called neuraminic acid," while "sialic acids" was suggested as group name for the acylated neuraminic acids (for example: N-acetylneuraminic acid, N-glycolyl neuraminic acid, N-O-diacetylneuraminic acid). Gottschalk (263) supposed that neuraminic acid would be a condensation product of pyruvic acid and hexosamine. According to Kuhn & Brossmer's (259) and Comb & Roseman's (264) work on the constitution of the neuraminic acid, it possesses the following formula (II). According to Comb & Roseman (264) N-acetylneuraminic acid is enzymatically cleaved to pyruvic acid and N-acetyl-D-mannosamine by an enzyme of *Clostridium perfringens.*

$$
\begin{array}{c}
\text{COOH} \\
\mid \\
\text{C}\!-\!\!-\text{OH} \\
\mid \\
\text{CH}_2 \\
\mid \\
\text{HO}\!-\!\!-\text{C}\!-\!\!-\text{H} \\
\mid \\
\text{H}_2\text{N}\!-\!\!-\text{C}\!-\!\!-\text{H} \\
\mid \\
\text{O}\!-\!\!-\text{C}\!-\!\!-\text{H} \\
\mid \\
\text{H}\!-\!\!-\text{C}\!-\!\!-\text{OH} \\
\mid \\
\text{H}\!-\!\!-\text{C}\!-\!\!-\text{OH} \\
\mid \\
\text{CH}_2\text{OH}
\end{array}
$$

II
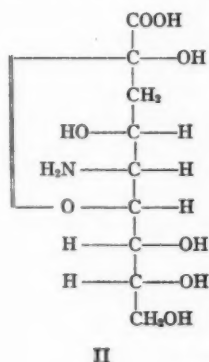
N-Acetyl- and N-glycolylneuraminic acids are widely distributed substances occurring in the mucoproteins, sometimes in amounts of more than

10 per cent. N-acetylneuraminic acid in its linkage to the mucin appears to be the biological active receptor substance of the human erythrocytes for influenza virus (265 to 268). Because of the lack of space, more cannot be said here on this matter; the reader may consult some recent reviews (269 to 272).

CEREBROSIDES

With paper chromatographic methods relative large amounts of cerebrosides were found recently in lipide fractions of different kinds of viruses (273). Studies on psychosine by Sakagami (274) confirmed the earlier findings of Carter & Greenwood (275) that the galactose is attached to the terminal carbon atom of sphingosine. Compounds related to cerebrosides such as N-benzoylsphingosine glucosides were synthesized by Proštenik & Krvavica (276). The α-oxytetracosenoic acids of brain cerebrosides consist of two isomeric forms, namely Δ^{15}- and Δ^{17}-n-α-oxy-tetracosenic acid, as it was shown by Klenk & Faillard (277). A cerebroside sulfuric acid ester first isolated by Blix (278) was shown by Thannhauser et al. (279, 280) to possess the sulfuric acid at the sixth carbon atom of galactose. Recently a simple procedure for the isolation of brain sulfatides was described by Lees (281). Radin et al. (282) have found that when C^{14} galactose and S^{35} sulfate are used no metabolic breakdown of sulfatides takes place.

GANGLIOSIDES

In 1935 Klenk (283) reported the finding of cerebrosidelike substances which could be found in the crude sphingomyeline fractions isolated from the brain in cases of Niemann-Pick's disease. Further investigations showed that this lipide (substance X) was accumulated specially in brain of one kind of lipidoses: the Tay-Sachs idiocy. After methanolysis, it was possible to isolate a hitherto unknown polyoxymonoamino acid (called neuraminic acid) by Klenk (284) in the form of its methoxy derivative (288). This substance is responsible for the characteristic color reaction with Bial's orcinol reagent, resulting a violet pigment soluble in amyl alcohol. The color reaction was used by Klenk & Langerbeins (285) as a quantitative estimation for neuraminic acid or gangliosides. The method was later on modified by Böhm et al. (286) and by Svennerholm (287). The neuraminic acid-containing lipide was found to occur not only in brain in cases of Tay-Sachs disease, but also in normal brains, particularly in its gray matter, although in much smaller amounts. It was isolated for the first time by Klenk (288), who proposed the name "ganglioside." Stearic acid, sphingosine or a sphingosinelike base, hexoses (mainly galactose besides a little glucose), and neuraminic acid were found as split products in a molar ratio of approximately 1:1:3:1. The neuraminic acid occurs in the brain gangliosides as N-acetyl derivative (289 a, b, c), while the gangliosides of horse erythrocytes (289c, 290, 291) and the bovine spleen (289c) contain N-glycolylneuraminic acid. Brante (292), applying paper partition chromatography in

studies on water-soluble split products of brain lipides, obtained a very distinct spot which gave a positive reaction with the Elson-Morgan reagent and with ninhydrin and which was moving at practically the same rate as chondrosamine. Thus Blix *et al.* (293, 294) reported the isolation of chondrosamine from gangliosides hydrolysates. Very soon these findings were confirmed by Klenk (295) who found a molar proportion of hexoses and aminohexoses of approximately 5:1. A preliminary formula for gangliosides was given by the same author (296). However, it is supposed by different authors that brain gangliosides are highly polymerized substances. Yamakawa *et al.* (297) concluded this from the physico-chemical properties of the aqueous solution. A gangliosidelike substance, called strandin, was isolated by Folch *et al.* (298, 299) from gray matter of brain. The finding that this substance contains no neuraminic acid proved to be erroneous (300, 301), and it seems that this substance is identical with brain ganglioside (302, 303, 304). A "mucolipide" which contains 5 per cent of amino acids, besides sphingosine, neuraminic acid and chondrosamine, was isolated from ox brain by Rosenberg & Chargaff (305). The impossibility of liberating the substance from the amino acids leads the authors to conclude that this mucolipide would represent a hitherto unknown entity. Very recently Bogoch (304) investigated the structure of brain gangliosides by a quantitative stepwise hydrolytic procedure. The author reported a minimum molecular weight of 250,000, although his preparation was not contaminated with proteins or peptides. However, the high molecular weight found with the ultracentrifugal method in aqueous solution may also be explained by a simple aggregation of smaller lipide molecules. Klenk (288) based his isolation method on solubility relations, including a separation of gangliosides from the last traces of sphingomyeline on alumina oxide columns while Svennerholm (306) described a method using a cellulose column, and Bogoch (304) isolated his gangliosides by the distribution methods of Folch *et al.* (298, 299). Immediately after the isolation of gangliosides from brain by Klenk (288), gangliosides were also obtained from ox spleen (307) and detected in human spinal cord (308). Yamakawa & Suzuki (260) found a ganglioside (called "hematoside") in the stroma of horse erythrocytes. It is a hexosamine-free substance also found by Klenk *et al.* (309, 310) and contains fatty acids, sphingosine, hexoses (galactose and glucose), and neuraminic acid in a molar ratio of 1:1:2:1 (261). Neuraminic acid occurs here in its glycolyl derivative (289c, 290, 291). Very small amounts of hexosamine containing gangliosides can be detected in the stroma of bovine (311, 312, 313) and horse (310) erythrocytes.

OTHER GLYCOSPHINGOLIPIDES

Investigating the cerebrosides of ox spleen, Klenk & Rennkamp (307) isolated a "cerebroside fraction" which consisted of a cerebroside-like substance with the molar ratio of fatty acid-sphingosine-hexose (galactose and glucose) as 1:1:2. Since 1951 numerous substances have been found which

seem to be closely related to cerebrosides in that they do not contain neuraminic acid. On the other hand, they do not belong to the cerebrosides since they possess more than one mole of hexoses and since often amino sugars are present in the molecule. Such cerebrosides have also been found by Klenk *et al.* (309, 310) in horse erythrocytes. Finally, a hexosamine-containing, but neuraminic acid-free glycolipide was obtained for the first time by Klenk & Lauenstein (314, 311) from human erythrocyctes. This finding was confirmed by Yamakawa & Suzuki (315). In the meantime some of these hexosamine-containing glycolipides could be detected from the erythrocytes of various animals (309, 310, 312, 313, 316). They differ mainly in that they contain sometimes glucosamine, sometimes galactosamine, or both in varying quantities. The same variations were observed by Yamakawa *et al.* (317) from erythrocytes of the individual blood groups. The authors therefore concluded that the ratios of sphingolipide-hexosamine components show some correlation with specifity.

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CHEMISTRY OF AMINO ACIDS AND PEPTIDES[1,2]

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The rapid progress in the field under review has continued unabated during the past year. Among the highlights are the synthesis of several biologically important peptides, as for example hypertensin and gramicidin-S, and the structural determination of a number of others. No doubt this is only the beginning of a major advance which has been made possible by the mastering of methodological problems.

Space limitations and lack of knowledge or interest on part of the reviewer have caused some aspects of the subject, e.g., the metabolic and the physiochemical, to be omitted from this treatment.

ANALYTICAL METHODS

Contributions under this heading fall naturally into two groups, one on structural and the other on compositional analysis.

Structural Analysis

Terminal amino acids.—The situation with regard to C-terminal analysis is not yet as satisfactory as that for N-terminal analysis, and consequently most contributions deal with the former problem.

Bradbury (1, 2) has made a careful study of Akabori's hydrazinolysis procedure with applications to insulin, lysozyme, and wool proteins, as well as model peptides, and reports more clear-cut results after incorporation of hydrazine sulphate in the reaction mixture. The yield of C-terminal amino acid(s) was generally satisfactory, although not quantitative, but the C-terminal amino acid(s) was always contaminated by small amounts of non-C-terminal amino acids, presumably attributable to hydrolytic side reactions. These by-products should not obscure the result for smaller protein molecules, but might do so for larger molecules. The same difficulty applies to C-terminal determinations by the ester reduction method (3, 4).

The problem of unspecific cleavage, to which Crawhall & Elliott (5) have drawn attention, has been made the subject of a searching study by Chibnall and his colleagues (6, 7, 8). The conclusion, in their own words, is that "the procedure is not recommended as a reliable one for proteins but it may be of use with peptides of low molecular weight." This series of

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[1] The survey of the literature pertaining to this review was concluded in November 1958.

[2] The following abbreviations are used: ACTH for adrenocorticotropic hormone; Ahypro *for* D-allohydroxyproline; Dimeleu *for* L-β,N-dimethylleucine; Hypic *for* 3-hydroxy picolinic acid; Meval *for* N-methylvaline; MSH for melanocyte-stimulating hormone; Phesar *for* L-α-phenylsarcosine; Sar *for* sarcosine.

papers contains a wealth of valuable information on the analysis of proteins, to which space does not permit reference.

Chappelle & Luck (9) have proposed a method of determining the free α-carboxyl groups in amino acids, peptides, and proteins, based on decarboxylation with N-bromosuccinimide (10) and manometric estimation of the released carbon dioxide. The results obtained with a number of proteins seem very promising. This reaction could probably also have applications in the identification of C-terminal residues.

Methoxycarbonyl chloride has been investigated as a reagent for N-terminal analysis [Chibnall & Spahr (11)] but has not been found to possess any advantages over those reagents already existing.

Amino acid sequences.—The clarification of the mechanism of the phenyl isothiocyanate degradation (12)—which incidentally has made the relationship to the older phenyl isocyanate method seem rather remote—has also cleared the way for an extension of its scope. The reaction has three steps:

$$\text{Phenylthiocarbamyl peptide} \xrightarrow{\text{acid}} \text{5-thiazolinone} + \text{peptide residue} \tag{1}$$

$$\text{5-Thiazolinone} \xrightarrow{\text{water}} \text{phenylthiocarbamyl amino acid} \tag{2}$$

$$\text{Phenylthiocarbamyl amino acid} \xrightarrow{\text{acid}} \text{3-phenyl-2-thiohydantoin} \tag{3}$$
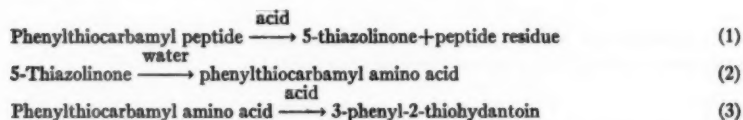
The first and second steps (1, 2) are fast, whereas the third (3) is slow. It has been found in the reviewer's laboratory that side reactions can be reduced to insignificant levels by limiting the exposure of the phenylthiocarbamyl peptide to acid to the time necessary for the completion of Reaction 1. The 5-thiazolinone is subsequently separated from the peptide residue and converted to the corresponding thiohydantoin in a separate operation. This modified technique has been found, in an actual case, to permit up to 13 consecutive degradations with a repetitive yield averaging over 80 per cent (13).

The use of leucine aminopeptidase for sequential analysis from the N-terminus holds out considerable promise. An improved method for the preparation of the enzyme from swine kidney, in a form free from endopeptidase activity, and exploratory work on its use in sequence determinations have been reported [Hill & Smith (14)]. The designation of the enzyme, leucine aminopeptidase, seems to be a misnomer, since its discrimination with regard to amino acid side chains is remarkably small for a proteolytic enzyme, and it is actually this property that allows the enzyme to penetrate deeply into a peptide chain. Results from digestion experiments with various proteins as substrates are in full accord with the assumed specificity of the enzyme. The problem of elucidating the amino acid sequence is, in principle, reduced to that of determining the relative rates at which the amino acids appear in the hydrolysate. The power of this approach is indicated by the actual determination of six amino acid residues at the

N-terminus of the B-chain of insulin. An extension of the procedure would seem to depend heavily on the accuracy of the methods used for determining the rates at which the amino acids are released. Another valuable feature is the stereospecificity of the degradation as it permits the location of D-amino acid residues (15).

Selective fragmentation of peptides.—Troublesome side reactions, particularly with tryptophan, are encountered in the oxidative cleavage of protein disulphide bonds with performic acid, and this has prompted exploration of the reductive route of cleavage. Moore *et al.* (16) have recently reported promising results with reduction by sodium borohydride and have overcome the earlier limitations resulting from incomplete reduction [Edman & Diehl (17)]. The thiol groups are protected against reoxidation through carboxymethylation. Fully reduced and carboxymethylated products of ribonuclease and chymotrypsinogen have been prepared. Swan (18), in a preliminary communication, has drawn attention to the many potentialities, in structural as well as in synthetic work, of the oxidative cleavage of disulphides brought about by sulphite and cupric ions (19):

$$RSSR + 2SO_3^{2-} + 2Cu^{2+} \rightarrow 2RSSO_3^- + 2Cu^+ \qquad (4)$$
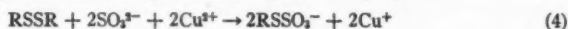
The sulphocysteinyl peptides are, on the one hand, sufficiently stable for isolation and, on the other, readily regenerated to cysteinyl peptides by reducing agents, e.g., thiols. A more detailed report is awaited with interest.

A preliminary fragmentation of a peptide is necessary when it is too long and unwieldy to be handled by the present techniques of sequence analysis. A high degree of selectivity is desirable in order to reduce the number of fragments, and this requirement is largely met by certain proteolytic enzymes. However, an even higher degree of selectivity seems to be offered by a reaction discovered by Patchornik *et al.* (20, 21). The oxidation of the peptide chain with N-bromosuccinimide brings about nuclear bromination of the tryptophyl residues, which then in several steps leads to cleavage of the C-tryptophyl bonds:

(5)
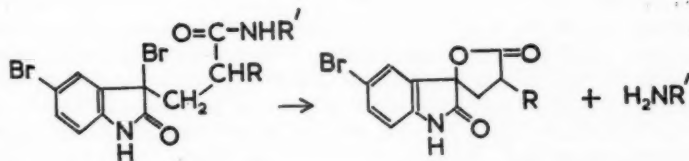
COMPOSITIONAL ANALYSIS

The problems of amino acid analysis have in the last decade received an enormous amount of attention and with most gratifying results. Indeed, it may well be asked if any other analytical problem has been more successfully solved. Most of the credit for this remarkable achievement goes to the

group at the Rockefeller Institute. If the problem still receives attention, and the stream of publications certainly indicates that this is so, this continuing interest may be ascribed to the need felt for simpler (and less costly) solutions.

Moore, Stein and their associates have crowned their achievements by the publication of an improved and automatic procedure for amino acid analysis (22, 23). The improved performance derives from the use of a finer and more carefully graded preparation of the sulphonated polystyrene resin [cf. Hamilton (24)]. Two columns are needed, one for the neutral and acidic amino acids, and one for the basic. With equipment for the automatic recording of the ninhydrin colour of the effluent, the complete analysis of as little as one to two mg. of protein hydrolysate can be accomplished within 24 hr. A technique described by Simmonds (25) operates on the same principle, but allows the simultaneous, automatic operation and recording of an assembly of columns.

Sjöquist has based his analytical procedure on a different principle. The amino acids of the protein hydrolysate are quantitatively converted to 3-phenyl-2-thiohydantoins (26), and these are separated on a partition column (27). The thiohydantoins have a high ultraviolet absorption, and this can be automatically recorded in the effluent (28). This technique requires only 150 to 300 μg. of protein, and other merits are speed and accuracy. However, it has not yet been possible to resolve all amino acids in a single operation. The various uses of phenyl thiohydantoins in protein analysis have been surveyed in a lecture (29).

The extremely powerful gas chromatographic procedure has also been applied to amino acid analysis. The solution of the crucial problem of converting the amino acids to volatile products has in one instance been attempted by esterification [Bayer *et al.* (30)], and in another by oxidation to aldehydes [Zlatkis & Oro (31)]. However, certain amino acids are unlikely to give rise to sufficiently volatile derivatives, and the general applicability of the principle is therefore still in doubt.

Amino acid analysis has entered the ultramicro range with a contribution by Whitehead (32), as the procedure developed by him requires only 2 μg. of protein for a complete quantitative analysis. This analytical feat has been achieved through a combination of the techniques of double isotope dilution, paper chromatography, and paper electrophoresis (33). Many histochemical problems should be within reach of this method.

Several papers (34 to 39), deal with amino acid determination by paper chromatography, paper electrophoresis, or a combination of the two. These methods derive their main attraction from technical simplicity, but their accuracy, although sufficient for many purposes, usually suffers from the fact that paper is not as inert a support as could be desired. A certain improvement in the resolution of the basic amino acids can be achieved through the use of so-called ion exchange papers (40, 41). Papers with

these properties are now commercially available, and are also readily prepared, e.g., by succinylation of ordinary filter paper (41).

The determination of individual amino acids, free or protein-bound, is the subject of many publications. In a colour reaction claimed to be specific for hydroxyproline, the imino acid is oxidized with chloramine-T to a product which gives a colour with p-dimethylaminobenzaldehyde. The reaction can be applied both to paper chromatograms for location and to solutions for quantitative determination (42, 43). The combined use of the ninhydrin and isatin reagents for locating and estimating hydroxyproline on paper chromatograms has also been recommended (44, 45). Modifications of the colorimetric 1-nitroso-2-naphthol method for tyrosine (46) and the spectrophotometric method for tyrosine and tryptophan (47) are described.

An interesting approach to the so far unsolved problem of estimating asparagine and glutamine in proteins has been made by Chibnall et al. (48). The method is based on the fact that asparaginyl and glutaminyl residues remain unaffected by reduction of the esterified protein with lithium borohydride, and appear as aspartic and glutamic acid after hydrolysis, whereas C-terminal asparagine and glutamine under the same conditions are converted following hydrolysis, to γ-hydroxy-β-aminobutyric acid and δ-hydroxy-γ-aminovaleric acid, respectively. After hydrolysis the four amino acids are separated on a Dowex-50 column and estimated [Rees (49)]. Aspartic and glutamic acid form different products and do not interfere. The various sources of error have been evaluated with great thoroughness, but do not seem to be serious.

The widely used amperometric method for determining free or peptide-bound cysteine (50, 51, 52) has been subjected to a critical study by Burton (53), who has observed large and seemingly inexplicable deviations from theoretically expected values. The titration values were found to be highly dependent on the pH of the buffer medium, but this effect varied in an unpredictable way from one mercaptan to another. It may be mentioned that similar observations have been made in the reviewer's laboratory. Evidently, the situation requires further clarification before full confidence can be put into results obtained by this method. The substitution of ferricyanide ions for silver or mercuric ions has been suggested (54), but no attention appears to have been given to the effect of extraneous oxidizable material.

Miscellanea.—Meyniel et al. (55) have described a method for the separation and quantitative estimation of iodinated tyrosine and thyronine derivatives by ion exchange chromatography, and Kennedy (56) has made a comparison between the proteolytic and a modified alkaline hydrolysis method for the release of iodinated amino acids from thyroid tissue. Reduced glutathione can be estimated by ultraviolet spectrophotometry after reaction with fluoropyruvic acid (57). Dipeptides are conveniently determined through their intensely coloured oxygenated cobalt complexes (58).

A nondestructive technique for locating amino acid spots on paper

chromatograms is based on the fluorescence of the spots after the paper has been treated with o-coumaric acid (59). Also, a new technique for revealing spots of phenyl thiohydantoins on paper chromatograms is reported (60).

The high frequency titration technique can be applied to the titration of amino acids in glacial acetic acid solution with perchloric acid, and non-interference from peptidic amino groups is claimed [Oehme (61)]. Kresze & Schmidt (62) have shown that trifluoroacetic acid can be substituted with advantage for glacial acetic acid in this titration.

The reported contamination of a commercial isoleucine preparation with *allo*isoleucine should serve as a warning (63).

PREPARATIVE METHODS

The field of peptide synthesis has been covered recently in several excellent reviews [Cook & Harris (64); Goodman & Kenner (65); Schwyzer (66); Wieland & Heinke (67); Young (68)] One of these (65) contains a tabular index of the peptides and peptide derivatives synthesized in the period between 1950 and 1956—in all approximately 2000. With the earlier index of this kind (69), there is now virtually a complete coverage of reports on synthetic peptides, and the authors of this Herculean work have earned the gratitude of all workers in the field. The present exposition can thus restrict itself to the latest developments.

A necessary preliminary to the establishment of a peptide linkage is the "activation" of one or the other of the components entering the bond. Several new procedures have been suggested for the activation of the carboxyl component. A variation of the procedure using the mixed anhydrides of N-acylamino acids and phosphoric acid is proposed by Cramer & Gärtner (70, 71). The activating agent is diethyl-(α-ethoxy-β-carbethoxy) vinyl phosphate:

$$\text{Cbo} \cdot \text{NH} \cdot \text{CHR} \cdot \text{COOH} + \underset{\underset{\text{O} \cdot \text{C(OEt)} : \text{CH} \cdot \text{CO} \cdot \text{OEt}}{|}}{\text{PO} : \text{(OEt)}_2} \longrightarrow$$

$$\text{Cbo} \cdot \text{NH} \cdot \text{CHR} \cdot \text{CO} \cdot \text{O} \cdot \text{PO} : \text{(OEt)}_2 + \text{CH}_2 : \text{(CO} \cdot \text{OEt)}_2 \qquad (6)$$

No racemization was observed when these compounds were used in the synthesis of a few peptides, and the yields were generally high. Earlier observations by Wieland & Schneider (72) have led Anderson & Paul (73) to investigate further the use of active N-acylimidazoles in peptide synthesis. The N-acylimidazole is prepared by reacting the carboxyl component with N, N'-carbonyldiimidazole under anhydrous conditions, and the N-acylimidazole is afterward condensed with the amino component (free amino acid or ester). Racemization seems to be negligible if the condensation is performed at reduced temperature, and the yields are good. A detailed report is awaited with interest. A procedure which is somewhat reminiscent of the carbodiimide method (74) has been put forward by Stevens & Munk

(75). The N-protected carboxyl component is made to react with diphenyl-ketene-p-tolylimine to form a crystallizable adduct:

$$Cbo \cdot NH \cdot CHR \cdot COOH + (C_7H_7) \cdot N{:}C{:}C{:}(C_6H_5)_2 \rightarrow$$
$$Cbo \cdot NH \cdot CHR \cdot CO \cdot N(C_7H_7) \cdot CO \cdot CH{:}(C_6H_5)_2 \qquad (7)$$
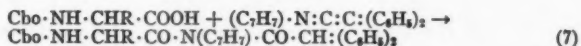
The active adduct is sufficiently stable to be stored. Condensation with an amino acid or peptide ester occurs at elevated temperature. The yields seem rather low, generally less than 50 per cent. No racemization was observed in the synthesis of N-carbobenzoxy-S-benzyl-L-cysteinyl-L-tyrosine and of N-carbobenzoxy-L-asparaginyl-S-benzyl-L-cysteine. Heslinga & Arens (76) have established peptide linkages by refluxing in ethyl acetate the N-protected carboxyl component and the C-protected amino component in the presence of ethyl α-chlorovinyl ether or ethyl α,α-dichloroethyl ether. They envisage, as intermediate, the acid chloride of the carboxyl component. If this is correct, it could cause difficulties because of the well-known tendency of compounds of this type to undergo side reactions (65, p. 475). A third route to the preparation of active p-nitrophenyl esters of amino acids and peptides using di*cyclo*hexylcarbodiimide has been indicated (77). A proposal for the carboxyl activation of tosyl amino acids by their conversion to oxazolidones (78) leaves some doubt about its value, since it has not been tried for racemization.

Earlier expectations that activation of the amino component would rule out racemization in the carboxyl component during coupling have not been substantiated (79, 80). Grassmann *et al.* (81, 82), using the "phosphorazo" method (83), found that the optical configuration was completely retained only if the carboxyl component was an amino acid derivative and that a dipeptide derivative suffered partial racemization in its C-terminal residue. This again serves to emphasize that with "asymmetrically unsafe" procedures, it is generally preferable to have only one amino acid in the carboxyl component. A procedure by Schramm & Wissmann (84), in which formation of the peptide bond is brought about by diethylphosphite and phosphorous pentoxide, appears also to proceed through an activation of the amino component.

The observations on the remarkable aminoacyl insertion (*Aminoacyl-Einlagerung*) reaction of Brenner have been further extended by the Swiss group (85 to 89). The base-catalyzed reaction follows the general scheme:

$$H_2N \cdot CHR' \cdot CO \cdot O \cdot R \cdot CO \cdot NH_2 \rightarrow HO \cdot R \cdot CO \cdot NH \cdot CHR' \cdot CO \cdot NH_2;$$
$$H_2N \cdot CHR'' \cdot CO \cdot O \cdot R \cdot CO \cdot NH \cdot CHR' \cdot CO \cdot NH_2 \rightarrow \qquad (8)$$
$$HO \cdot R \cdot CO \cdot NH \cdot CHR'' \cdot CO \cdot NH \cdot CHR' \cdot CO \cdot NH_2, \text{ etc.}$$
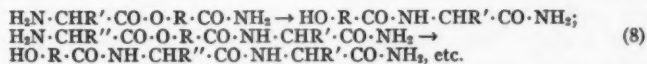
The only restriction on the nature of R seems to be that it should contain one or two carbon atoms. The fact that serine and threonine fulfill this condition could have a significant bearing on the biosynthesis of peptide chains, and the more so since the rearrangement has now been demonstrated to go with full retention of asymmetric configuration. The prospects of

using the reaction as a regular method of peptide synthesis are being explored.

Some remarks of a more general nature may be in place here. A profusion of methods are at present available for the establishment of peptide bonds. Several of these leave little to be desired in the way of versatility and operational convenience, and they constitute marked improvements over the acid chloride and azlactone methods of the not so distant past. However, looking ahead to the synthesis of the large structures now amenable to structural determination, the prospects do not seem so bright. At least two obstacles stand out clearly, racemization and low yields. As regards the former we are not concerned with the more or less complete racemization produced in some methods, since for all biological purposes they are no methods at all, but rather with the small, and consequently easily overlooked, degrees of racemization. The ruinous effects of using even slightly inhomogeneous amino acid preparations (with only a few per cent of the enantiomorph) in the synthesis of large peptides have been pointed out by Greenstein (90), and the same applies, of course, to racemization produced during synthesis. One therefore seems justified in calling for more rigorous tests of optical homogeneity in synthetic work on peptides. Obviously, the optical rotation loses relevance to this question as the number of asymmetric centres increases. The biological methods, using either microorganisms or purified enzyme preparations, are undoubtedly superior for the purpose. For a discussion of these methods the reader is referred to the review by Greenstein (90). However, attention should be drawn to the great potential possibilities of the enzyme leucine aminopeptidase, which now can be prepared in a sufficient degree of purity (14). It has already been used, and with revealing results, for checking the optical homogeneity of a synthetic peptide related to the melanocyte-stimulating hormone [(15); cf. p. 86 of this review].

Secondly, in the synthesis of long peptide chains, the question of yield becomes a serious problem, as a simple calculation shows. Consider a hypothetical case, where a peptide with 32 amino acid residues is synthesized by two different methods of establishing peptide bonds, one with 80 per cent and the other with 40 per cent yield in the unit operation. Under most favourable conditions, i.e., symmetrical pairing of intermediate peptides, the former method would return in the final product 33 per cent of the starting amino acids against only 1 per cent in the latter procedure. It is doubtful that with present techniques even the lower figure is attainable, and efforts to improve this situation are strongly indicated.

Much attention has been given to the designing of new protective groups. It seems unlikely that any reagent, whatever merits it may have, will supplant the classical benzyloxycarbonyl chloride, if for no other reason than that this would mean the scrapping of much of the earlier literature on peptide synthesis. The merits of new reagent would lie less in any general

superiority, than in a special reactivity which would distinguish it from the already established reagents. This applies especially to the conditions for the removal of the protective group, since the synthesis of complex peptides usually calls at one stage or another for the selective unmasking of a particular function without interference with the protection of other functions.

McKay & Albertson (91) have investigated the properties of various oxycarbonyl chlorides [$R \cdot O \cdot CO \cdot Cl$; R = t-butyl;p-methoxybenzyl;$cyclo$pentyl;$cyclo$hexyl;(diisopropyl)-methyl] as N-protective reagents and have favoured the $cyclo$pentyloxycarbonyl group. It is readily removed by hydrogen halides but not by hydrogenolysis. The t-butyloxycarbonyl group has essentially the same reactivity, and Anderson & McGregor (92) have suggested an elegant way of introducing this group using t-butyl-p-nitrophenyl carbonate. A seemingly successful attempt to revive the formyl group, a "racemizer" because of the tendency for oxazolone formation, as a N-protective group has been made by Sheehan & Yang (93). Apparently the mild conditions of the carbodiimide procedure minimize the favourable properties of the formyl group. It is fairly resistant to saponification, but is readily removed by acid alcoholysis (94). Schwyzer *et al.* (95) have proposed p-phenylazo-benzyloxycarbonyl chloride ($C_6H_5 \cdot N : N \cdot C_6H_4 \cdot CH_2 \cdot O \cdot CO \cdot Cl$) and p-(p'-methoxyphenylazo)-benzyloxycarbonyl chloride ($CH_3 \cdot O \cdot C_6H_4 \cdot N : N \cdot C_6H_4 \cdot CH_2 \cdot O \cdot CO \cdot Cl$) for amino protection. These reagents form coloured derivatives which should be of considerable advantage, especialy in the purification of more complex peptide derivatives, and should then outweigh the disadvantage that the decomposition products resulting from the removal of the protective groups are not volatile.

Several contributions are concerned with the synthetic problems arising from the reactivities of particular amino acid side chains. In the classical procedure of du Vigneaud both S- and N-protective benzyl groups are removed in one operation through reduction with sodium-liquid ammonia. However, situations often occur where the selective unmasking of either the amino or the thiol functions are called for. The synthesis of insulin, to quote one case, would hardly be possible without methods for this. Sheehan & Yang (96) and King *et al.* (97) reacted cysteine with acetone to form a thiazolidine:

(9)
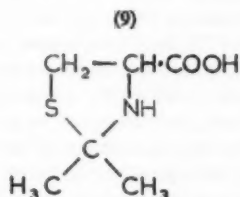
The imino group is still reactive, and can be used for extension of the chain or be protected, e.g. by formylation (93), as the case may be. The isopropyli-

dine group is readily removed by mild acid hydrolysis. No racemization is observed. Holland & Cohen (98) have had some success with the use of dihydropyran (99) as a S-protective group:

(10)
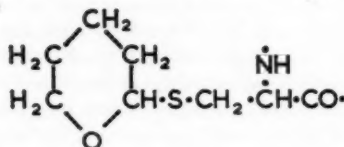
The 2-tetrahydropyranyl group can be removed by aqueous silver nitrate leaving the cysteine derivative as its silver mercaptide. The fact that benzyl and p-nitrobenzyl groups can be differentially hydrogenolysed has been taken advantage of by Berse et al. (100) for the selective protection of amino and thiol functions.

Usually the protection of the hydroxyl group of serine is unnecessary in peptide synthesis, but should this problem arise, use of the benzyl derivative should be considered, particularly since Grassmann et al. (101) have published an elegant synthesis of O-benzyl serine from readily available starting materials [cf. also (102)]. The same grouping is also advocated (103) for the protection of the tyrosine hydroxyl group, claimed to be necessary in the phosphorazo method. Debenzylation is done by catalytic hydrogenation in both cases.

The prospects for the synthesis of arginine peptides should have brightened considerably after the report by Zervas et al. (104) on synthetic routes to $N_\alpha, N_\omega, N_\omega$-tricarbobenzoxy-, $N_\alpha N_\omega,$- and N_ω, N_ω-dicarbobenzoxy-, and N_ω-carbobenzoxy-L-arginine, since it is now possible to suppress as desired, any or all of the basic functions of this amino acid. Announcement of the first successful synthesis of arginylarginine followed speedily (105). Similarly, the preparation of N_α, N_{1m}-dicarbobenzoxy-L-histidine by Akabori et al. (106) and by Patchornik et al. (107) may be taken advantage of in the synthesis of histidine peptides. A new way to the selective protection of either the α- or the ε-functions of lysine has been opened by the observation that the N_α-trityl group of N_α, N_ϵ-ditrityllsyine methyl ester is preferentially hydrolyzed by acids [Amiard & Goffinet (108)].

Amino-protected aspartic acid cyclic anhydrides are valuable intermediates in peptide synthesis, and Weygand et al. (109) have shown that trifluoroacetic anhydride has the advantage of producing N-trifluoroacetyl aspartic acid anhydride in one operation, and with retention of optical configuration. It is claimed that with alcohols or amines the anhydride opens practically exclusively to the α-ester or α-amide respectively. An improvement in the separation of the α- and γ-ethyl esters of N-carbobenzoxy glutamic acid, using the differential solubility of the dicyclohexylamine

salts, is also described from the same quarter (110). A correction regarding the conditions for the preparation of N-tosylpyroglutamyl chloride has appeared (111). What was earlier (112) considered to be this compound has turned out to be N-tosyl glutamyl dichloride.

The current great interest in phosphopeptides attaches considerable importance to methods of phosphorylating the hydroxyl group of serine. For this purpose Fölsch & Mellander (113) and Fölsch (114) have reacted the N-carbobenzoxy protected amino acid or peptide ester with diphenyl phosphorylchloride. Subsequent hydrogenolysis produced in one step and in high yield the O-phosphorylated product. Zahn & Zürn (115) have described the first synthesis of peptides containing hydroxylysine and *allo*hydroxylysine. The δ-lactones of the amino acids were used as active intermediates.

The physical identification properties of some carbobenzoxy amino acids have been re-examined (116), but incidentally, no agreement as to the correct melting point of the L-phenylalanine derivative has been reached (117, 118). An experience probably frequently encountered, but until now unpublished, is that excess nitrite in the azidation of peptides containing tyrosine causes nitration of the phenol group. (119).

The synthesis of several mono- and difunctional polypeptides has been described. Poly-L-histidine with 15 to 50 residues was produced through polymerization of N_{1m}-benzyl-N-carboxy-L-histidine anhydride and subsequent removal of the protective benzyl group in sodium-liquid ammonia. This polymer shows a marked tendency to form insoluble complexes with various metal ions [Patchornik *et al.* (120)] Poly-L-hydroxyproline ($n \approx 95$) was also prepared by the carboxy anhydride method, but with the novel feature that the "Leuchs" anhydride was obtained by reacting hydroxproline, suitably O-protected, with phosgene and silver oxide [Kurtz *et al.* (121)]. Solutions of the O-acetylated and O-tosylated poly-L-hydroxyproline derivatives displayed the interesting phenomenon of mutarotation, which has earlier been observed for poly-L-proline (122, 123). The synthesis of completely water-soluble poly-L-serine and poly-DL-serine preparations has been announced in a preliminary communication [Fasman & Blout (124)].

Polymerization can also be brought about simply by heat dehydration of an amino acid. However, as a method of synthesis it obviously suffers from the complete lack of control in the establishment of new bonds, and products obtained in this way (125, 126), although interesti.; from other points of view, will not be discussed here.

Amino acids.—The discovery that the incorporation of amino acids into proteins, *in vivo*, proceeds via mixed anhydrides of adenylic acid and the amino acids (127, 128) has stimulated several attempts to synthesize compounds of this kind. Evidence that the desired product has been obtained is not easily secured, mainly because of the great lability of these compounds. That usual test for "activated" carboxylic acids, viz., formation of hydroxamic acids with hydroxylamine, can be misleading is shown by the fact that valine esterified to the ribose of adenylic acid also gives a

positive test [Wieland *et al.* (129, 130); cf. Raacke (131)]. The proposed methods [Berg (132); Castelfranco *et al.* (133); Wieland & Jaenicke (130)] all use di*cyclo*hexylcarbodiimide for establishing the linkage between amino acid and adenylic acid.

A number of papers deal with the preparation of amino acids either by synthesis or by isolation from natural sources. It is hardly possible to discuss these contributions in a limited space, and they will therefore only be enumerated. Preparative methods for the isolation of amino acids in larger quantities from protein hydrolysates have been described [Buchanan (134); Selim *et al.* (135, 136, 137)]. The following papers are concerned with synthesis of L-lysine [Brenner & Rickenbacher (138)], DL-hydroxy-lysine and DL-*allo*hydroxylysine [Zahn & Zürn (139)], DL-serine and DL-isoserine [Gundermann & Holtmann (140)], γ-aminobutyric acid and α,γ-diaminobutyric acid [Talbot *et al.* (141)], and DL-carnitine [Mazzetti & Lemmon (142)]. Another group of papers is concerned with the preparation of the enantiomorphs of lysine [Murachi (143)], 5-hydroxy-tryptophan [Morris & Armstrong (144)], serine [Losse & Augustin (145)], valine and leucine [Sakurai (146)], phenylalanine and γ-phenyl-α-aminobutyric acid [Tanaka & Izumiya (147)] and of α-amino acids in general [Parikh *et al.* (148)].

AMINO ACIDS AND PEPTIDES OF NATURAL ORIGIN

This treatment excludes the *in vitro* degradation products of proteins since, although undisputedly of natural origin, such products are more appropriately discussed in the context of the larger structures from which they are derived. Also excluded are reports on the natural occurrence of peptide materials, which at present are not sufficiently identified as compounds of definite composition.

MICROORGANISMS

Peptides.—Bruckner & Kovács (149) have recently summarized the work leading to the clarification of the structure of the bacterial capsular polypeptides, an achievement to which they and their associates have contributed so richly. A most readable account of the chemistry of the bacitracin and cephalosporin antibiotics has been given by Abraham (150).

The immunospecific capsular substances of *Bacillus anthracis* and *Bacillus subtilis* have been known for some time to be polyglutamic acids. The controversial question of the mode of linkage between the glutamic acid residues seems now to have been definitely resolved in favour of the γ-polyglutamic acid alternative. Furthermore, results obtained through reduction of the esterified *Bacillus subtilis* polypeptide with lithium boro-hydride (cf. p. 73) together with titration data make the presence of branched or cyclic peptide chains very unlikely [Chibnall *et al.* (151)]. It had earlier been established that the anthrax polypeptide is made up entirely

of D-glutamic acid, whereas the *Bacillus subtilis* polypeptide contains both D and L isomers. Studies on synthetic γ-polyglutamic acids containing either the L or D forms exclusively, or L or D isomers alternately, have now demonstrated that the two latter products react with anthrax immune serum, whereas the pure L form is devoid of activity [Bruckner *et al.* (152, 153, 154)].

The polypeptide antibiotics constitute a strange collection of compounds. Bizarre forms of amino acids of "unnatural" optical configuration linked in devious ways seem to be the rule rather than the exception. A recent acquisition is etamycin, which is produced by a *Streptomyces* species. Sheehan *et al.* (155), in a very elegant work, have shown it to be a macrocyclic peptide lactone containing eight amino acids of which four have not been encountered earlier in nature, namely 3-hydroxypicolinic acid (hypic), L-α-phenylsarcosine (phesar), D-*allo*hydroxyproline (ahypro) and L-β,N-dimethylleucine (dimeleu). The structure is shown in Fig. 1. The creation of an N-terminal amino acid residue, required for stepwise degradation, was accomplished in an inspired way, through catalytic hydrogenation of the pyridine ring of the hydroxypicolinyl group to a piperidine ring. Saponification of the peptide lactone afforded the desired straight chain peptide.

The identification of the chromophoric group of the actinomycins with 3-amino-1,8-dimethyl-phenoxazon-(2)-dicarboxylic-(4,5) acid has permitted the establishment of the complete structures for actinomycin-C_3 and -χ_1 [Brockmann & Muxfeldt (156); Bullock & Johnson (262)], and alternative structures for actinomycin-χ_2 and -$\chi_{o\beta}$ [Brockmann & Manegold (157)] (Fig. 2).

The structure of amidomycin from a *Streptomyces* species has also been clarified [Vining & Taber (158)]. It is a 24-membered cyclic structure composed of only two elements, four D-valine and four D-α-hydroxyisovaleric acid molecules, and the two residues alternate as do the peptide and ester bonds joining them. There is an obvious relationship to valinomycin, the differences being that in the latter L-lactyl groups have substituted for the D-α-hydroxyisovaleryl groups in two places, and that the valyl residues have the L configuration (159).

The structure of bottromycin from *Streptomyces bottropensis* is gradually emerging [Waisvisz *et al.* (160, 161, 162)]. The approximate empirical

$$\begin{array}{ccccc}
\text{hypic} & \rightarrow \text{thr} \rightarrow & \text{leu} & \rightarrow & \text{ahypro} \\
& & | & & \downarrow \\
& & \text{O} & & \text{sar} \\
& & | & & \downarrow \\
\text{phesar} & \leftarrow & \text{ala} & \leftarrow & \text{dimeleu}
\end{array}$$
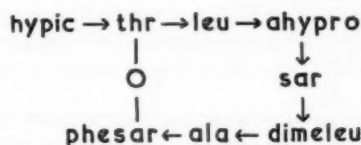
FIG. 1. Structure of Etamycin.
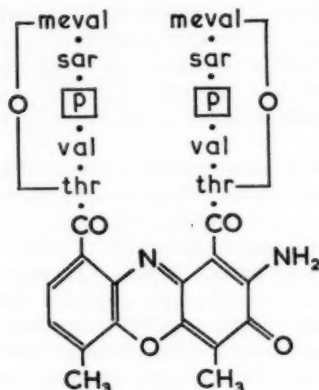
FIG. 2. Structures of the Actinomycins. Actinomycin-χ_1: P = proline. Actinomycin-χ_2: P = proline and γ-oxoproline. Actinomycin-$\chi_{\beta o}$: P = proline and hydroxyproline. Actinomycin-C_3: P = proline and *allo*isoleucine instead of valine. (Sarcosine is abbreviated "sar," and N-methylvaline "meval.")

formula, $C_{38}H_{57-61}N_7O_{8-7}S$, has been partly accounted for by the isolation of a peptide fragment

$$C_6H_5 \cdot CH(Met) \cdot CH(NH_2) \cdot CO \cdot NH \cdot CH(C_4H_2NS) \cdot CH_2 \cdot CO \cdot OMet \qquad (11)$$

composed of the two new amino acids α-amino-β-phenylbutyric acid and β-(2-thiazole)-β-alanine. Other components are glycine and valine. The methyl ester group seems to be essential for activity (163).

Duramycin [Shotwell *et al.* (164)], a polypeptide antibiotic from *Streptomyces cinnamomeus*, appears to be closely related to cinnamycin (165). On hydrolysis it yields the amino acids lanthionine, β-methyl-lanthionine, aspartic and glutamic acid, glycine, proline, valine, phenylalanine, and possibly ornithine and hydroxyproline. In the hydrolysate of an unnamed antibiotic isolated from a strain of *Paecilomyces*, Kenner & Sheppard (166) have reported the presence of a number of amino acids among which are L-leucine, β-alanine, α-amino*iso*butyric acid, D- or L-*threo*-β-hydroxyleucine, and a proline derivative which appears to be γ-methylproline. A preliminary report on the structure of circulin-A from *Bacillus circulans* has appeared [Koffler & Kobayashi (167)]. An interesting feature of its structure is that it contains a fatty acid, $(+)$-6-methyloctanoic acid, in addition to the amino acids.

The synthesis of gramicidin-S has been accomplished [Schwyzer & Seiber (168)]. The key intermediate in the synthesis of the cyclic decapeptide was the suitably protected amino acid sequence Val·Orn·Leu·Phe·Pro (L-L-L-D-L), which was prepared by the activated ester (*p*-nitrophenyl and cyanomethyl) procedures earlier developed in the same laboratory. Dimeri-

zation of the pentapeptide to the decapeptide was brought about by mor-pholinylethyl-3-*cyclo*hexylcarbodiimide (169). The crucial operation of cyclizing the decapeptide proceeded with remarkable ease after *p*-nitro-phenylester activation of the C-terminal carboxyl group and unmasking of the N-terminal amino group. In a series of stringent tests the synthetic product was found to be identical with gramicidin-S of natural origin. Er-langer *et al.* (170) have synthesized an acyclic decapeptide analogue of gramicidin-S with N-terminal valine, and D-tyrosine substituted for D-phenyl-alanine. Reports on the antibiotic activity of this peptide will be awaited with interest.

It is noteworthy that no peptide antibiotic so far has shown the structural regularity known from the amino acid sequences of proteins. The irregulari-ties may then lie in the structures or optical configurations of the amino acids or in the mode in which the amino acids are linked. As pointed out by Arnstein (171), a minimum requirement for antibiotic activity seems to be one amino acid residue with D configuration. The other striking feature is the cyclic structures. However, it should be recalled that synthetic straight chain analogues of gramicidin-S show a certain but low degree of antibiotic activity (172, 173). An obvious consequence of a cyclization is an increased rigidity of the structure, and any irregularity in the structure, e.g., a D-amino acid residue, would therefore become uniquely fixed in its relation to the whole structure. This must obviously be of importance in any sterically con-ditioned interaction with other structures, e.g., a specific receptor in the bacterium.

An earlier communication [Cartwright (174)] on the isolation and tenta-tive identification of a metabolic product from *Serratia* organisms, ser-ratamic acid, has now been followed by a report on its structure and synthesis (175). The fatty acid component has been rigorously identified, and the proposed structure is N-(D-3-hydroxydecanoyl)-L-serine.

Tritsch & Wooley (176) have isolated a new peptide with strepogenin activity from an enzymic digest of insulin, and determined its structure:

$$(H \cdot Leu \cdot Val \cdot Cys \cdot Gly \cdot Glu \cdot Arg \cdot OH)_2 \qquad (12)$$
$$S$$
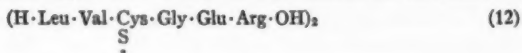
The list of peptides with strepogenin activity is now quite long, and the structural similarities between them are not very apparent. This makes one think that, if there is a common structural denominator, this is probably several metabolic steps removed from the structures studied.

PLANTS

The vegetable kingdom is a happy hunting ground for investigators in search of new amino acids. The findings are usually isolated in the sense that only rarely can a biological function or a metabolic relationship be assigned to them.

Peptides.—The complete structure of the polypeptide evolidine, earlier

isolated from the leaves of *Evodia xanthoxyloides* (177), has now been
announced [Law *et al.* (178)]. Degradative studies have shown it to be a
cyclic heptapeptide of the structure:

$$\overline{\text{Ser} \cdot \text{Phe} \cdot \text{Leu} \cdot \text{Pro} \cdot \text{Val} \cdot \text{Asp} \cdot \text{Leu}} \quad \text{(all L)} \qquad (13)$$
$$\text{NH}_2$$
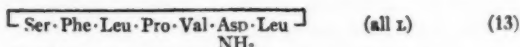
Virtanen & Ettala (179) have isolated a tripeptide from *Juncus con-glomeratus,* for which the structure γ-glutamylvalylglutamic acid is claimed.
The identification of the γ-glutamyl bond rests only on the observation that
it is easily hydrolysed by acids, and would need further confirmation. The
optical configurations of the constituent amino acids are not reported.

The isolation of a dipeptide, γ-L-glutamyl-S-methyl-L-cysteine from the
nonprotein fraction of beans has been independently reported from two
laboratories [Rinderknecht *et al.* (180, 181); Morris & Thompson (182)].
The presence of the corresponding sulphoxide could also be demonstrated.
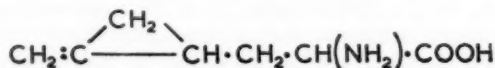
FIG. 3. Structure of hypoglycin.

It should be recalled that S-methyl-L-cysteine and its sulphoxide have been
encountered earlier in plants and microorganisms (183, 184, 185).

Amino acids.—The current great interest in hypoglycin, the hypoglycemic
substance in the fruits of *Blighia sapida* (186, 187), is reflected in the fact
that its structure is now reported from six laboratories [Anderson *et al.*
(188); Ellington *et al.* (189); v. Holt & Leppla (190); Renner *et al.* (191);
de Ropp *et al.* (192); Wilkinson (193)]. The substance has been identified
as an α-mino acid of unusual configuration (Fig. 3). Unfortunately, no
reference can be made here to the interesting arguments leading to this
structure. L-configuration is probable at C-2, but the steric configuration at
C-4 remains undetermined. Hypoglycin occurs also in bound form as a
dipeptide with glutamic acid. The synthesis of hypoglycin has been an-
nounced in a preliminary report [Carbon *et al.* (194)].

Virtanen & Matikkala (195, 196) have isolated a sulphur-containing
amino acid from *Allium cepa* and *Allium sativum* and proposed the structure
3-methyl-1,4-thiazane-5-carboxylic acid-1-oxide. Its biological formation
from alliin [CH_2:$\text{CH} \cdot \text{CH}_2 \cdot \text{SO} \cdot \text{CH}_2 \cdot \text{CH(NH}_2) \cdot \text{COOH}$] could be envisaged
through cyclization over the amino group and the C-5. Two new amino acids,
S-(β-carboxyethyl)-L-cysteine and α-amino-β-ureidopropionic acid (named
albizziin), have been discovered in the seeds of a *Mimosacea* species,
Albizzia julibrissin [Gmelin *et al.* (197)]. Burroughs (198) has isolated and
identified 1-amino-*cyclo*propane-1-carboxylic acid from perry pears and cider
apple, and its occurrence in berries of cowberry has also been reported
(199). This appears to be the second α-amino acid of natural origin without
an asymmetric centre. Homoserine in bound form has been found in the

fruits of cowberry and cranberry (200), and S-acetylornithine in several grasses of the family *Festuceae* (201).

Finally the occurrence of three amino acids should be mentioned because of antiquity rather than novelty. Heijkenskjöld & Möllerberg (202) have demonstrated in anthracite (estimated age: 250 million years) the presence of glycine, aspartic, and glutamic acid in an over-all concentration of about 1 per cent. These amino acids could very well be the remnants of the original plant proteins, but their notoriously facile formation by nonbiological routes should be kept in mind (203, 204, 205).

ANIMALS

Peptides.—The literature pertaining to the chemistry of the anterior pituitary hormones has recently been reviewed by Li (206, 207, 208).

The synthesis of peptides closely related to equine Ileu^5-hypertensin-II (angiotonin, angiotensin) has been announced from two laboratories [Rittel *et al.* (209); Schwarz *et al.* (210)]. The natural product has the structure (211):

$$H \cdot Asp \cdot Arg \cdot Val \cdot Tyr \cdot Ileu \cdot His \cdot Pro \cdot Phe \cdot OH \qquad (14)$$

The Swiss group chose to synthesize the asparaginyl rather than the aspartyl peptide. In spite of this modification, the synthetic product had a specific activity on the blood pressure comparable to that of the natural product. The American group attempted the synthesis of unmodified hypertensin-II, but the final product, although it had the expected specific activity, was not completely homogeneous in countercurrent distribution studies. The fact that the over-all yield in these syntheses was less than 1 per cent indicates a limitation in present synthetic procedures (cf. p. 76).[3]

The structure of porcine ACTH [Bell *et al.* (212, 213); White & Landmann (214)] has been supplemented by the structures of the corresponding hormones from sheep [Li *et al.* (215)]. and cattle [Li *et al.* (216)] [Table I (15)]. A comparison between these structures shows that the species differences reside in the sequence comprising the amino acid residues 25 to 33 from the N-terminal. It is then interesting that degradation experiments with the porcine hormone (213) have shown this part of the structure to be unessential for the adrenocorticotropic activity, as measured by the usual tests.

Geschwind *et al.* (217) have now isolated from bovine pituitary glands the melanocyte-stimulating hormone, β-MSH, and determined its structure. It differs from the corresponding porcine hormone (218, 219) in that a serine residue is substituted for a glutamic acid residue in position-2 [Table I]. In addition to standard methods of peptide analysis, i.e., phenylisothiocyanate and carboxypeptidase degradations and N-terminal fluorodinitrobenzene analysis, leucine aminopeptidase degradation (14)

[3] The Swiss laboratory has subsequently reported in full the syntheses of the bovine hormone, Val^5-hypertensin-II, and its decapeptide precursor, Val^5-hypertensin-I [Schwyzer *et al.* (260, 261)].

was used. The enzymatic degradation stopped at the Gly·Pro bond, which thus falls outside the specificity of this enzyme.

The structure of the second melanocyte stimulating hormone, α-MSH from pork pituitaries [Lee & Lerner (220); Lee (221)], has been announced by Harris & Lerner (222) [Table I]. The sequence comprising positions 4 to 10 is identical with that of β-MSH and ACTH, and a connection between this structure and melanocyte stimulating activity now seems established. Furthermore, its whole sequence is contained in the N-terminus of ACTH, with the modifications that the N-terminal amino group is acetylated [Harris (223)] and the C-terminal carboxyl group amidated. The masking of the former group could be of importance for the activity (223), since it is known that destruction of the N-terminal serine of ACTH by periodate oxidation enhances the intrinsic melanocyte stimulating activity of this hormone (224).

Several attempts to synthesize peptides related to ACTH and MSH have been described. Hofmann *et al.* (15, 225, 226) have synthesized the peptides 18 to 21 (Table I). Degradation with leucine aminopeptidase was used extensively to check the optical homogeneity of the synthetic products, and it is noteworthy that in one instance (15) considerable racemization was observed after coupling of two peptides with carbodiimide. Peptide 19 (Table I), which contains the sequence of seven amino acids common to α-MSH, β-MSH, and ACTH, displayed a significant melanocyte stimulating activity, although the specific activity was only a few per cent of that of α- and β-MSH. The lower activity could arise from the substitution of a glutamine residue in the synthetic product for the glutamic acid residues in the natural products (225), but it should be remembered that ACTH has only a weak effect on melanocytes. Boissonnas & Guttmann (227) have made preliminary announcement of complete synthesis of α-MSH, but details are not available. The synthesis of the amino acid sequence 33 to 39 of bovine ACTH [22, Table I] has also been reported in brief [Oertel (228)].

A series of papers by du Vigneaud and co-workers (229 to 232) deals with improvements in the synthesis of arginine and lysine vasopressin. A troublesome side reaction in earlier synthesis has been that C-terminal asparagine and glutamine, when activated by carbodiimides (74) or alkyl pyrophosphites (233), tend to undergo a dehydration reaction and form what appears to be the corresponding ω-nitriles. By avoiding this reaction it has now been possible to synthesize arginine vasopressin with the same specific activity as the natural product.

New attempts have been made to correlate structure and biological activity through the synthetic approach. Ressler & du Vigneaud (234) have synthesized the isoglutamine analogue of oxytocin (24, Table II). The most obvious steric effect of the substitution is an enlargement of the disulphide ring, and this might explain the observed lack of oxytocic activity. In the oxytocin analogue synthesized by Guttmann *et al.* (235), ring enlargement was brought about by insertion of a second tyrosine residue (25, Table II). This peptide, also devoid of oxytocic activity, is as a matter of fact an

oxytocin inhibitor. However, a very high ratio of inhibitor to oxytocin seems to be required for this effect.

The question of the existence of a posterior pituitary protein hormone carrying the oxytocic and vasopressor activities [van Dyke *et al.* (236)] was much in debate some years ago, but a resolution was not reached. It has now been revived by a communication wherein is described a protein complex of this kind used with advantage for the early stages in the purification of oxytocin and vasopressin [Acher *et al.* (237)]. It seems to the reviewer that the question of the "carrier protein" merits a reinvestigation with the powerful aid of present-day techniques in view of its great physiological importance. Two other papers deal with the purification of the posterior pituitary hormones by ion exchange chromatography [Ward & Guillemin (238); Light *et al.* (239)].

The interesting observation that the ocular lens contains sulphur-free analogues of glutathione [Waley (240)], has now been supplemented from the same laboratory by reports on structures (241), synthesis (242), and biosynthesis (243) of these compounds. One of the peptides, ophthalmic acid, has been identified as γ-glutamyl-α-aminobutyrylglycine, and the other, norophthalmic acid, as γ-glutamylalanylglycine. The occurrence of S-sulphoglutathione ($GSSO_3H$) has also been reported in a preliminary note (244). No physiological role has yet been ascertained for these compounds, but it is more than a conjecture to assume that they are of importance for the optical properties of the lens.

Amino acids.—Remarkably high concentrations of cystathione (up to 55 mg. per cent) have been discovered in human brains, whereas only negligible amounts were found in a number of other species [Tallan *et al.* (245)]. No definite explanation for this interesting fact is at present offered. The identity of γ-aminobutyric acid, also present in high concentration in the central nervous system, with the inhibitory neuron transmitter, Factor I of Florey [Florey *et al.* (246, 247)], is at present considered (248, 249). Many neurophysiological effects of Factor I and γ-aminobutyric acid are indistinguishable, e.g., the inhibition of stretch receptors in crayfish, but the parallelism is not complete (250), and more doubt arises by the failure of paper chromatography to demonstrate the presence of free γ-aminobutyric acid in brain extracts containing Factor I (251). It is possible that γ-aminobutyric acid is only part of an inhibitory transmitter complex (250). The metabolic precursor of neural γ-aminobutyric acid is, as would be expected, glutamic acid [Roberts *et al.* (252)].

Citrulline is not considered a constituent of animal proteins, and therefore a report to the contrary arouses considerable interest [Rogers (253)]. The inner root sheath protein(s) of hair follicles is claimed to contain as much as 6 per cent of this amino acid [Rogers & Simmonds (254)]. It is not a constituent of keratin, and its physiological function is obscure.

The search for likely biological pathways in the formation of thyroxine using model systems has been continued by Pitt-Rivers & James (225), and it has been found that a high yield of thyroxine is obtainable from N_ϵ-(N-

TABLE I

STRUCTURES OF ACTH, α-MSH, AND SYNTHETIC PEPTIDES RELATED TO THESE COMPOUNDS

Bovine ACTH

(15) H·Ser·Tyr·Ser·Met·Glu·His·Phe·Arg·Try·Gly·Lys·Pro·Val·Gly·Lys·Lys·Arg·Arg·Pro·Val·Lys·Val·Tyr·Pro·Asp·Gly·Glu·Ala·Glu·Asp·Ser·Ala·Glu·Ala·Phe·Pro·Leu·Glu·Phe·OH
$$NH_2$$

Porcine α-MSH

(16) Ac·Ser·Tyr·Ser·Met·Glu·His·Phe·Arg·Try·Gly·Lys·Pro·Val·NH₂

Bovine β-MSH

(17) H·Asp·Ser·Gly·Pro·Tyr·Lys·Met·Glu·His·Phe·Arg·Try·Gly·Ser·Pro·Pro·Lys·Asp·OH

(18) H·Ser·Tyr·Ser·Met·Glu·His·Phe·Arg·OH
$$NH_2$$

(19) H·Ser·Met·Glu·His·Phe·Arg·Try·Gly·OH
$$NH_2$$

(20) H·His·Phe·Arg·Try·Gly·OH

(21) H·His·Phe·Arg·Try·Gly·Lys·Pro·Val·NH₂

(22) H·Glu·Ala·Phe·Pro·Leu·Glu·Phe·OMet
$$OEt$$
$$OEt$$

TABLE II
Structures of Oxytocin and Synthetic Analogues

Oxytocin

$$H \cdot Cys \cdot Tyr \cdot Ileu \cdot Glu \cdot \ Asp \cdot \ Cys \cdot Pro \cdot Leu \cdot Gly \cdot NH_2 \qquad (23)$$

$$H \cdot Cys \cdot Tyr \cdot Ileu \cdot Isoglu \cdot Asp. \ Cys \cdot Pro \cdot Leu \cdot Gly \cdot NH_2 \qquad (24)$$

$$H \cdot Cys \cdot Tyr \cdot Tyr \cdot Ileu \cdot Asp \cdot \ Glu \cdot \ Cys \cdot Pro \cdot Leu \cdot Gly \cdot NH_2 \qquad (25)$$

acetyl-diiodotyrosyl)-N_a-acetyl lysine under conditions which were well within the physiological. The pertinence of such observations to the physiological process is not obvious, since the spontaneity of a reaction may not be a merit in a homeostatic mechanism. Roche *et al.* (256) have isolated 3,3'-diiodo- and 3, 3',5-triiodothyronine in substantial quantities from hydrolysates of porcine thyroglobulin, and thus furnished confirmative evidence for the natural occurrence of these compounds. A remarkable deiodination reaction of thyroxine and other iodophenols has been announced in a preliminary report by Tata (257). A full report will be awaited with great interest.

The occurrence of ergothioneine and its sulphur-free analogue, herzyinie, in the crustacean *Limulus polyphemus* has been reported [Ackermann & List (258)], and the metabolic relationship is being investigated. Heath & Toennis (259) have shown that ergothioneine in acid solution is reversibly oxidizable to the disulphide. The occurrence of this reaction *in vivo* is an interesting possibility.

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THE STRUCTURE OF PROTEINS[1,2]

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Studies of the amino acid sequence of proteins have as their ultimate aim the correlation of these structural features with biological activity. This field is expanding rapidly, partly because of the availability of methods which permit unequivocal determination of structure and partly because a knowledge of structure permits precise studies of the effects of chemical alterations upon biological activity of proteins.

Two noteworthy and interrelated concepts appear to dominate present attempts to understand the biological activity of the proteins. One is the fact that intact primary structure is not essential for activity of many proteins. Although this has been known for some time in the case of antibodies, only recently has it been possible to demonstrate this for certain hormones and enzymes. The second concept is that of the active site, i.e., the realization that only a part of the molecule is essential for activity which, in favorable cases, can be modified or labeled by specific reagents. These approaches thus permit, on the one hand, isolation of active units smaller than the intact protein and thereby facilitate the study of the larger proteins, and, on the other hand, determination of the sequence of isolated, inactive units bearing a specific label whose introduction has resulted in the inactivation of the protein. Notable progress has been made in both types of studies.

It is necessary to add a word of caution regarding some of the current optimism in the use of these approaches. First of all, our knowledge of protein structure has been obtained only by degradative methods and proof of structure must, as in the case of simpler molecules, be demonstrated by unequivocal synthesis, a possibility which is remote at the present time. Secondly, it has been found that intact "folded" structure is essential for biological activity of all enzymes which have been carefully studied. It is thus apparent that the active site involves much more than amino acid sequence alone and that we shall have to understand three-dimensional structure before it is possible to approach a complete solution of structure-activity relationships.

It is desirable to state at the outset that much effort must still be devoted to methodology at various levels of investigation. In the first part of the

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[1] The survey of the literature pertaining to this review was completed in October 1958.

[2] The following abbreviations are used: ATP for adenosine triphosphate; DFP for diisopropyl phosphorofluoridate; DIP for diisopropylphosphoryl; DNP for dinitrophenyl; TCA for trichloroacetic acid.

review attention will be devoted to methods for obtaining pure proteins and peptides, methods of amino acid analysis, and methods of determining end groups and sequence. In addition, a key feature of work with larger poly-peptides and proteins involves opening disulfide bridges to prepare stable derivatives which are susceptible to proteolytic digestion. Digestion with proteolytic enzymes remains the primary method of degrading larger sub-stances to smaller peptides; however, this will not be reviewed at present since the enzymes presently used for this purpose have been adequately discussed on a number of occasions. The second portion of this review de-scribes studies with certain proteins selected either because some informa-tion has been obtained regarding structure or because progress is being made in studying structure-function relationships.

The *Symposium on Protein Structure* (1) provides important summaries of information up to the summer of 1957.

AMINO ACID ANALYSIS

The development of chromatographic methods of amino acid analysis has had a profound effect upon protein chemistry in the last decade. These methods are now so good that other factors which may limit the precision of an analysis have become increasingly important, e.g., the method of hy-drolysis and the determination of nitrogen content [Chibnall *et al.* (1a)].

Although the intact protein may be used for estimation of some amino acids, e.g., tryptophan, analyses are usually performed on hydrolysates ob-tained by heating the protein at 110°C. in an excess of redistilled $6N$ HCl in a sealed, evacuated tube for at least 24 hr. Under these conditions certain amino acids undergo destruction which can be demonstrated by time studies. Destruction of serine and threonine has long been recognized (2) and has been observed with ribonuclease (3), papain (4), carboxypeptidase (5), papaya lysozyme (6), α-chymotrypsinogen (7), and the hemoglobins studied by Stein *et al.* (8). Other amino acids which may be destroyed on acid hydrolysis are aspartic acid, glutamic acid, arginine, lysine, tyrosine, and proline (3, 4, 5). It should be emphasized that destruction is assumed on the basis that lower yields of amino acids are obtained if acid hydrolysis be prolonged. In the case of threonine and serine, there is no doubt that de-struction does occur since this has been demonstrated with synthetic mix-tures (3). In some cases the increase in ammonia associated with prolonged hydrolysis has been equal to the amount of serine and threonine lost (4, 5). Although destruction of other amino acids may occur, it may be noted that a resistant peptide may chromatograph in the same manner as an amino acid. In a 70 hr. hydrolysate such a peptide may no longer be present, thus giving rise to an apparent decrease in the yield of an amino acid. Inasmuch as apparent destruction varies, each protein is an individual problem, and one cannot formulate any fixed factors to correct for losses associated with hydrolysis.

Smith, Stockell & Kimmel (4), as well as Wilcox, Cohen & Tan (7),

have used a linear extrapolation based on zero order kinetics to estimate destruction, whereas Hirs, Stein & Moore (3) have assumed that first order kinetics are applicable. Wilcox, Cohen & Tan (7) have noted that neither type of correction may be valid, especially during the initial stages of hydrolysis.

Another factor that influences the accuracy of an analysis is the variation in susceptibility of certain peptide bonds to acid hydrolysis. Sanger (9) has discussed the relative stability and lability of peptide bonds in a review of the older literature. More recently, Harris, Cole & Pon (10) have studied a specific group of peptides in this regard.

The over-all effect of the relative stability of certain peptide bonds is that prolonged hydrolysis is necessary to obtain complete release of such amino acids as valine and isoleucine and of any amino acids bound to these residues. Present information (7) indicates that maximal yields of amino acids are obtained after 70 hr. hydrolysis. We have emphasized the problem of hydrolysis because this now limits the accuracy of the analysis, when the best available methods are used. It is apparent that some investigators do not consider these factors worthy of attention, since only a few laboratories routinely perform analyses after more than one hydrolysis time.

Methods of separation and estimation of amino acids: Ion exchange chromatography.—The procedures of Moore & Stein (11, 12) have been valuable tools for quantitative amino acid determination for some years. These methods involve chromatography on ion exchange columns and color development with a ninhydrin reagent. Moore, Spackman & Stein (13) have reported a new ion exchange procedure which improves the precision of the analysis. Spackman, Stein & Moore (14) have adapted this procedure to an automatic recording apparatus which permits a complete analysis in 24 hr. While eluate from the ion-exchange columns passes through a continuous length of tubing, it is mixed with ninhydrin, heated for 15 min. at 100°; and the color is measured photometrically and recorded automatically. The authors modestly claim a precision of 100 ± 3 per cent for loads from 0.1 to 3.0 µM of each amino acid. Because of the stable base line, the instrument has a sensitivity equal to, if not better than, paper chromatographic methods for detection of amino acids.

Simmonds (15) also has applied automation to the ion exchange columns of Moore, Spackman & Stein. His apparatus collects fractions of eluate, performs the reaction with ninhydrin, and records separately the color intensity of each eluted fraction. Simmonds' apparatus is probably less expensive and can be adapted easily to other types of chromatography. In addition, it is possible to perform several analyses simultaneously if proper recording equipment is available.

Hamilton (16) has recently reviewed methods for chromatographic separation of amino acids and related compounds.

Paper chromatography.—There have been many attempts to apply paper chromatography to the quantitative estimation of amino acids. The pro-

ponents of these methods point out that it is theoretically possible to perform more analyses in a given period of time than with column methods. In general, paper chromatographic methods fall into several categories which are listed below with their variants and with selected, recent applications of these methods.

(i) Direct separation of the amino acids: (a) Two-dimensional paper chromatography (17, 18, 19); (b) Ionophoresis in one dimension, followed by paper chromatography in a second dimension (20, 21); (c) Repeated development of a chromatogram in one dimension with drying between each solvent application (22).

(ii) Quantitative determination of the amino acids: (a) Develop color on paper with ninhydrin; measure intensity of spot with densitometer or, alternatively, measure area of spot (17, 18, 19); (b) Elute amino acid from paper, develop color with ninhydrin and measure directly by colorimetry (17, 18, 19); (c) Develop color on paper, transfer pattern to photographic film, and perform densitometry (22).

(iii) Methods involving derivatives of amino acids: (a) Convert amino acids to dinitrophenyl derivatives; separate by two-dimensional chromatography, elute spots, and measure intensity of yellow color (23); (b) Convert to N-acetyl amino acids with C^{14}-acetic anhydride; separate by two-dimensional paper chromatography, elute, and measure radioactivity (21); (c) Convert to "pipsyl" derivative, chromatograph, elute, and measure radioactivity (17, 18, 19).

(iv) Isotope dilution methods: Dilute amino acids in hydrolysate with known amounts of C^{14}-labeled amino acids; separate by two dimensional chromatography, elute spots, and count (24).

The major problem associated with paper chromatographic methods is the actual estimation of each amino acid present. There are almost as many methods for accomplishing this as there are investigators who have studied the problem. If quantitation is performed by densitometry, then the consistency of the paper, the size of the spot, and the conditions of color development with ninhydrin all influence the results. If quantitation is performed by elution of the spot, either before or after treatment with ninhydrin, the problem of ammonia contamination is encountered. Numerous methods have been devised to circumvent this difficulty (17, 18, 19). Kornberg & Patey (25) have described the use of a cation exchange resin for removal of ammonia from eluates.

An extensive application of quantitative paper chromatography is to be found in the work of Keil, Šorm, and their colleagues (26 to 31), who use unidimensional chromatography and photograph the chromatogram after staining with ninhydrin. The blackening of the developed film is measured with a microphotometer. This method has proved useful for determination of the composition of small peptides. It has also been applied to proteins; however, Šorm's (26) analysis of chymotrypsinogen does not agree with that reported by Wilcox et al. (7). The precision of Šorm's methods is

estimated to be about ±5.0 per cent based on his analyses of serum albumins (26).

The method of Levy (23) which involves the dinitrophenyl amino acids has been used for the analysis of several polypeptide hormones (32, 33, 34). However, this method demands very careful standardization, since the yields of DNP-amino acid from the original reaction with fluorodinitrobenzene are not strictly quantitative.

Whitehead (21) has recently described a method wherein the amino acids are labeled with C^{14} by conversion to the N-acetyl derivative; the mixture of labeled amino acids is then diluted with known amounts of H^3-labeled amino acids. The mixture is resolved by ionophoresis and chromatography, the spots are combusted and counted both for C^{14} and H^3. The potential precision of the method is ±2 to 5 per cent for as little as 2 μg. of protein. The method appears to be very laborious.

In general, quantitative paper chromatography does not seem to offer the precision necessary for sequence studies of large peptides or proteins. However, the methods are certainly useful for analysis of small peptides. The experience of the authors has been that the time and expense required to develop confidence in quantitative paper chromatographic methods are greater than those required to put into operation the ion exchange methods.

Amino acid analyses have been performed on the following proteins by ion exchange chromatography: human hemoglobins, A, C, E, F (8, 35), bovine myoglobins (36), α-chymotrypsinogen (7), bacterial amylase (37), ATP-creatine transphosphorylase (39), horse heart cytochrome-c (40, 41), phycocyanin and phycoerythrin (42), soybean hemagglutinin (43), T_2 and T_3 bacteriophages (44), elastoidin (45), collagen fractions (46), fish collagen and gelatin (47), and wool fractions (48). Paper chromatographic methods have been used for analysis of phosphoglucomutase (38), soluble feather keratin (49), vertebrate nucleohistones (50), serum albumins, chymotrypsinogen and trypsinogen (26). Microbiological and chemical methods have been employed for analysis of dephosphorylated casein (51) and proteins of barley grain (52).

CYSTINE AND CYSTEINE OF PROTEINS

The cystine and cysteine residues in proteins present special problems with regard to both studies of amino acid composition and studies concerned with the disulfide bonds of proteins. The two aspects are closely related, but the methodology may be quite different.

The method most extensively used today for estimation of total cystine or half cystine in proteins is that of Schram, Moore & Bigwood (53). This procedure involves oxidation of the protein with performic acid at low temperatures, followed by hydrolysis, and estimation of cysteic acid after separation by chromatography on cation or anion exchangers.

Recently, new methods for breaking disulfide bonds in proteins have been described, and these may be of considerable value for determining the total

half-cystine content of proteins. Moore *et al.* (54) have reduced several proteins with sodium borohydride in the presence of 8 *M* urea. The reduced protein is then allowed to react with iodoacetic acid to convert cysteine residues to S-carboxymethyl cysteine residues. After hydrolysis, this derivative can be estimated by chromatographic methods. Any destruction that occurs during hydrolysis can be estimated by time studies. Only minimal alkylation of other amino acids occurs during the reaction with iodoacetic acid; however, methionine recovery may be slightly low. Application of this method to chymotrypsinogen gave a corrected yield of S-carboxymethyl cysteine corresponding to 99 per cent of theory.

Cleavage of disulfide bonds in proteins.—Performic acid oxidation was originally applied to insulin by Sanger (55) as a method for separating the A and B chains. Since then this method has been used with ribonuclease (56), lysozyme, (57), papain (58), and bovine plasma albumin (59), to mention a few proteins. The major disadvantage of this procedure is that tryptophan is destroyed during the oxidation and a number of products, including kynurenine, are formed (54). Furthermore, chlorination of tyrosine can occur unless careful precautions are taken to remove residual peroxide (60). Repeated freeze-drying may not remove residual peroxide in all cases, but this can be accomplished by addition of a trace of catalase (61).

In order to preserve tryptophan, reductive methods for breaking disulfide bridges appear to offer considerable promise. The method of Moore *et al* (54) described above is very promising in this regard. Similar methods have been published (62, 63) in which reduction of the protein in the presence of a denaturing agent (e.g., urea or guanidine) is performed with thioglycolate. These methods suffer from the disadvantage that the reducing agent is itself a thiol, thereby making it very difficult to estimate the extent of reduction. Furthermore, subsequent alkylation requires removal of excess thioglycolate or use of a large excess of alkylating agent. Moore *et al.* (54) have reported that, when this procedure is applied to ribonuclease, tyrosine and methionine residues, as well as cysteine, are affected.

The reaction of disulfides with sulfite has received some attention recently (64, 65, 66). Swan (65) has discussed this reaction and has pointed out that the presence of cupric ions rapidly drives the reaction to completion (67). For the cleavage of disulfide bonds in proteins the reaction is very specific, but its usefulness is limited, at the moment, because of the difficulty of ascertaining the extent of reduction of disulfides and because the cysteine-S-sulfonate formed is converted to cystine during acid hydrolysis. However, this reaction presents a number of useful features: it is reversible; it can be used to incorporate S^{35} into a protein; it can be used to prepare mixed disulfides; and, potentially, it could be adapted to specific cleavage of peptide bonds at cysteine residues. Neurath, Dixon & Pechère (68) have successfully applied Swan's method to the study of both trypsinogen and chymotrypsinogen; their results will be considered below under the specific proteins.

Free sulfhydryl groups in proteins.—Hellerman & Chinard (69) have reviewed methods for estimating free thiol groups in proteins and have noted the advantages and limitations of each. Since the publication of this review, Boyer (70) has described a spectrophotometric procedure which utilizes *p*-chloromercuribenzoate.

The major problem associated with determination of sulfhydryl is that different answers are obtained with different reagents. Since the use of *p*-chloromercuribenzoate generally gives the highest values, it has been assumed that this compound is better able to react with hindered thiol groups (69).

The amperometric estimation of sulfhydryl groups with silver or mercury has been widely used recently, largely because of the work of Kolthoff and several associates (71 to 74), Benesch & Benesch (75, 76), and Benesch, Lardy & Benesch (77). However, some observers have experienced difficulty with these methods. For example, Sluyterman (78) found that thioglycolic acid, cysteine HCl, and cysteine ethyl ester HCl, when titrated amperometrically with Ag^+ in either ammonia or tris hydroxymethylaminomethane buffer, combined with 1.3 to 1.6 moles of Ag^+ per thiol group, whereas glutathione combined in the expected ratio. Staib & Turba (79) reported similar findings for cysteine. These discrepancies have not been accounted for fully, but they do cast some doubt as to the specificity of the method. This problem is apparent in considering the results reported for hemoglobin. Amino acid analysis of human hemoglobin A by Stein *et al.* (8) gave 4.9 residues of half cystine (as cysteic acid) per mole of protein, and amperometric titration gave a value equivalent to 4.6 thiol groups per mole. On the basis of the same type of titration, Ingram (80), Benesch *et al.* (77), Murayama (81), and Hommes *et al.* (82) found eight free sulfhydryl groups per mole of denatured hemoglobin. Obviously eight sulfhydryl groups cannot arise from, at most, six residues of half cystine. It is known that a thiol can bind more than one silver atom [Allison & Cecil (83); Cecil & McPhee (84); Sluyterman (78*)*]. A discussion of this problem is also presented in a symposium on hemoglobin (85).

The amperometric methods can, however, be exceedingly useful, as demonstrated by the studies of Katchalski, Benjamin & Gross (86) and of Kolthoff *et al.* (87) on the denaturation of serum albumin.

METHODS OF END GROUP ANALYSIS

Chemical methods of amino-terminal analysis.—There has been little change in the methodology of chemical determination of the N-terminal amino acids of polypeptides and proteins. The two principal methods continue to be the dinitrophenyl (DNP) method of Sanger (88) and the phenylthiohydantoin method of Edman (89). Both methods are now discussed in reviews (9, 90, 91).

The synthesis of some of the less common DNP amino acids has been described. Zahn and co-workers have reported the synthesis of N,N'-*bis*-

[2,4-dinitrophenyl]-*meso*-lanthionine (92), DNP derivatives of hydroxyly-sine (93), and O-DNP serine derivatives (94). Zahn & Pfannmüller (95) were unable to obtain imadazole-DNP-histidine, but could prepare di-DNP-histidine and N-α-DNP-histidine as have others (91). Heyns & Wolff (96) have reported a study of the conditions for dinitrophenylation in which gelatin was used as the model protein. They were able to select reaction conditions which gave the greatest yield of ε-DNP lysine and minimized nonspecific splitting of peptide bonds. Burchfield (97) has studied the molecular rearrangement which accompanies the reaction of cysteine with fluorodinitrobenzene.

Keil (30) has developed a technique for performing the dinitrophenylation, the extraction, and the hydrolysis in a single vessel. This technique eliminates the micro-extractions by using an ion exchange resin to separate the DNP-peptide or amino acid from reagents, and free amino acids. It appears to offer some advantages over older methods involving liquid-liquid extraction.

Recently, Ingram (98) has reported that upon catalytic reduction, 2,4-dinitrophenyl peptides will lactamize and split the N-terminal peptide bond. This provides a procedure similar to that reported by Holley & Holley (99) which appears to be satisfactory for stepwise degradation of simple peptides. Hörmann, Lamberts & Fries (100) have utilized catalytic hydrogenation to estimate the extent of dinitrophenylation of peptides. Quantitation is achieved by utilizing the Warburg apparatus to measure the hydrogen absorbed during reduction.

The Edman degradation has been applied by Hirs, Stein & Moore (101) to elucidate the amino acid sequence of peptides obtained from ribonuclease. Because of the difficulty encountered in identification and estimation of the phenylthiohydantoin these authors have utilized the subtractive method exclusively. After removal of the N-terminal amino acid by cyclization to form the phenylthiohydantoin, an aliquot of the degraded peptide is hydrolyzed and analyzed. The sequence of the peptide is deduced from the residues remaining after each degradative step.

Sjöquist (102) converts amino acids to phenylthiohydantoins for quantitative analysis of amino acid mixtures. In connection with this method, the synthesis and properties of the phenylthiohydantoins are described (102), as well as appropriate automatic, chromatographic methods for separation of mixtures (103, 104).

Enzymic methods of N-terminal analysis.—A procedure for obtaining highly purified leucine aminopeptidase (105, 106, 107) has permitted use of this enzyme for stepwise degradation of proteins and polypeptides from the N-terminus. The enzyme may act so rapidly that it is often difficult to obtain sequence information from quantitative analysis of the amino acids released. However, this type of information has been obtained by Hill & Smith (106) with the oxidized B-chain of insulin, by Geschwind *et al.* (108) with melanocyte-stimulating hormone, by Grassmann *et al.*

(109) with collagen, and by Ando *et al.* (110) with clupein and salmine.

The aminopeptidase also has been used in partial degradation studies where the remaining peptides have been separated and their composition determined. Sequence information has been derived from this subtractive method by Dixon *et al.* (111).

Inasmuch as the enzyme does not hydrolyze the ω-amide bonds of glutamine and asparagine, cleavage of a peptide by the aminopeptidase liberates these residues and permits direct identification of the dicarboxylic acids or their amides (106, 112).

Since the oxidized chains of insulin (106), hypertensin (113), and glucagon (112) can be completely hydrolyzed by the enzyme, these substances contain only L-amino acids.

Unfortunately, leucine aminopeptidase does not act on all native or even denatured proteins, despite the presence of suitable N-terminal residues; for example, human serum albumin is hydrolyzed only after performic acid oxidation (106). It is now recognized that carboxypeptidase behaves in a similar manner. α-Chymotrypsinogen has been reported to be devoid of a C-terminal group on the basis of the inability of carboxypeptidase to liberate amino acids from this protein (68). However, S-sulfochymotrypsinogen (68), prepared by treatment with cupric ammonium sulfite in the presence of 8 *M* urea, is attacked by carboxypeptidase-A. (The sequence is given below; see section on *Chymotrypsin,* p. 117.) Thus it should be emphasized that the inability of either aminopeptidase or carboxypeptidase to liberate amino acids from a protein is not evidence for the absence of terminal residues.

Chemical methods for C-terminal analysis.—A major deficiency in amino acid sequence methods is the lack of a completely satisfactory procedure for identification of carboxyl terminal residues.

Hydrazinolysis of proteins, as described by Akabori *et al.* (114, 115), and by Ohno (116), gives rise to amino acid hydrazides from all the amino acids in a protein except the C-terminal residues. Unfortunately, the procedure is not quantitative and large correction factors must be used (117, 118). In most cases, small amounts of free glycine, serine, glutamic acid, and aspartic acid are found in the hydrazinolysate (120, 121). Modifications of the original method have been described by Bradbury (118, 119), Heyns & Legler (120), by Kusama (121), and by Braunitzer & Schramm (122). End group determinations with hydrazine have been reported for serum albumins (121), cytochromes (123, 124), Taka-amylase A (125), collagen (120), insulin, lysozyme, and wool proteins (126).

Chibnall, Rees and their associates (127, 128, 129, 130) have reported studies of the hydride reduction method in a series of four papers. Their procedure depends upon esterification of the protein, reduction of the ester groups with $LiBH_4$, followed by hydrolysis and estimation of the amino alcohols isolated. The carboxy terminal amino acid should appear in the hydrolysate as the corresponding β-amino alcohol. In addition, glutamic

acid and aspartic acid residues should be esterified and reduced at the β or γ carboxyl group, in contrast to glutaminyl and asparaginyl residues which would appear as the acids in the hydrolysates. The present method suffers from the difficulties in obtaining complete esterification and from nonspecific peptide bond cleavage during esterification or reduction. With insulin these problems were not serious and excellent results were obtained, whereas with β_1-lactoglobulin the yield of alcohols resulting from peptide bond splitting was great enough to cause difficulties in interpretation of the results (Table I).

Chappelle & Luck (131) have described the use of N-bromosuccinimide

TABLE I

C-TERMINAL RESIDUES AND AMIDE DISTRIBUTION OF
INSULIN, LYSOZYME AND β_1-LACTOGLOBULIN (127,128)

	Insulin	Lysozyme	β_1-Lactoglobulin
C-Terminal Residues (moles/mole)	Ala 0.96 (1)* AspNH₂ 0.81 (1)	Leu 0.55 (1)†	Leu 1.2 (2)‡ Ala 3.9 Tyr 0.3 Gly 0.3
Amide Distribution (moles/mole)	AspNH₂ 3 (3) Asp 0 (0) GluNH₂ 3 (3) Glu 4 (4)	AspNH₂ 13 Asp 7 GluNH₂ 5 Glu 0	AspNH₂ 10 Asp 21 GluNH₂ 18 Glu 32

* Parenthetical values are those determined by sequence studies of Harris, Sanger & Naughton (313).

† Thompson (128a).

‡ Niu & Fraenkel-Conrat (117).

for decarboxylation of C-terminal amino acids in peptides and proteins. All α-amino acids yield 1 mole of CO_2 per mole of amino acid, with the exception of aspartic acid which yields 2 moles of CO_2 and cystine and tyrosine which yield 1.25 moles of CO_2. The method does not permit identification of the C-terminal amino acid. With a number of proteins the method gave the expected number of carboxyl groups where the C-terminal sequence was known (Table II). It should be noted, however, that Patchornik, Lawson & Witkop (132) have used N-bromosuccinimide under much the same reaction conditions to obtain peptide bond splitting at tryptophanyl residues.

Enzymic methods for C-terminal analysis.—Carboxypeptidase-A is used extensively to obtain sequence information at the carboxyl end of polypeptide chains. Unfortunately, the action of the enzyme is limited to certain C-terminal residues and is of little value when resistant peptide bonds are present (133). Tietze, Gladner & Folk (134) and Folk & Gladner (135, 136) have purified a carboxypeptidase-B, which appears to be specific for

C-terminal basic amino acids—lysine, arginine, and ornithine. Ando *et al.* (110) have used the enzyme to demonstrate C-terminal arginine in clupein and salmine. The protaminase studied by Weil & Telka (137) and Weil & Seibles (138) appears to be the same as, or very similar to, carboxypeptidase-B.

ISOLATION OF PEPTIDES AND PROTEINS

The progress being made in the study of protein structure has depended to a large extent on the development of methods for isolation and characterization of peptides and proteins. Although many established methods are currently in use, it is worth considering newer developments briefly.

Chromatography.—Separation of complex mixtures of peptides has been

TABLE II

C-TERMINAL GROUPS OF VARIOUS PROTEINS AS DETERMINED
WITH N-BROMOSUCCINIMIDE (131)

Protein	Reported	Found	Protein	Reported	Found
Insulin	2 (9)	2	α-Chymotrypsin	2 (242)	1
Chymotrypsinogen	1 (68)	1	δ-Chymotrypsin	2 (242)	2
α-Lactalbumin	1 (131)	1	Lysozyme	1 (128a)	1
β-Lactoglobulin	2 (117)	1	Trypsin	0 (242)	1
Papain	1 (287)	2			

achieved for the most part by chromatographic techniques. Schroeder (139), in a recent review, notes that optimal conditions for separating peptides must be found by trial and error. This review, as well as that of Moore & Stein(140), discusses the problems involved in peptide separations.

Schroeder *et al.* (141) separated 59 peptides from partial acid hydrolysates of white turkey feather quills by the use of both anion and cation exchange resins in conjunction with a gradient elution technique wherein both pH and temperature were varied. Certain mixtures of peptides obtained from these columns could be resolved by treatment of the mixture with fluorodinitrobenzene and subsequent chromatography of the dinitrophenyl derivatives on silicic acid–Celite columns. Sufficient yields were obtained to allow at least a partial identification of the isolated peptides and to account for about 30 per cent of the hydrolyzed protein as either amino acids or peptides.

Jollès, Jollès-Thaureaux & Fromageot (57, 142) have reported the separation of peptides obtained from both tryptic and peptic hydrolysates of egg white lysozyme. Resolution was achieved with Dowex-50X2 as the resin and a stepwise development involving changes in pH and ionic strength of the developing buffers. Seventeen peaks were obtained from the peptic hydrolysate whereas nine peaks were obtained from the tryptic hydrolysate.

However, tryptic hydrolysis of oxidized lysozyme and subsequent chromatography revealed the presence of 18 peptides. On the basis of composition and sequences of these peptides, a partial structure of lysozyme has been proposed (also see p. 121).

Ramachandran & Winnick (143) have described the isolation of several peptides found in desiccated hog pituitary gland. The technique of dinitrophenylation of the peptide mixture followed by chromatography of the DNP-peptides on Celite, silicic acid, and cellulose resulted in recognition of at least 31 different peptides, ranging in size from dipeptides to a polypeptide of about 84 residues.

Bromer & Behrens (144), as well as Raacke & Li (145), have reviewed older studies of the chromatography of the peptide hormones. Acher, Light & du Vigneaud (146) have used ion exchange chromatography for the final purification of oxytocin and vasopressin. Although the vasopressin obtained in their procedure was highly pure, the oxytocin required further purification. Peart (147) has described a partition chromatographic system using a supporting medium of diatomaceous earth for the isolation of hypertensin.

Feitleson & Partridge (148) describe the preparation of a new type of ion exchange reagent for peptide chromatography. Realizing that high molecular weight materials diffuse only slowly into ordinary resins, these workers applied a thin coating of low cross-linked polystyrene to a diatom skeleton preparation. After sulfonation, this material could resolve a large peptide obtained from tryptic digests of the B-chain of oxidized insulin.

Because location of peptide material after chromatography is often difficult, especially in the presence of amino acids, the differential ninhydrin procedure of Markovitz & Steinberg (149) should be useful.

Despite the successful chromatography of amino acids and peptides, it is only recently that there has been much progress with proteins. Nonetheless, the purification of small proteins on ion exchange resins, as well as more recent results with larger proteins on the cellulose ion exchange adsorbants, suggests that chromatographic procedures are now available for many types of proteins. A review of the literature up to 1957 can be found in the articles by Moore & Stein (140), Sober & Peterson (150), and Boman (151).

In order to simplify the discussion, Table III lists some of the more recent chromatographic separations of proteins and, although incomplete, it serves to illustrate many of the fundamental points that are now known. It is evident that, in addition to small basic proteins such as ribonuclease, lysozyme, and cytochrome-c, other types of proteins can be chromatographed —e.g., the acidic enzyme, gastricin, or larger proteins such as serum albumin. Furthermore, complex mixtures such as pancreatic juice, rat liver supernatant, and the alder pollen allergens can be partially resolved by chromatography. Although calcium phosphate has long been used for batchwise purification of proteins, its value as a chromatographic adsorbant has been described recently by Tiselius, Hjerten & Levin (181). Cellulose

TABLE III

CHROMATOGRAPHY OF PROTEINS

Protein	Chromatographic system*	Reference
Bovine pancreatic juice	DEAE-cellulose; XE-64; G.E.	(152)
Prostatic deoxyribonuclease	DEAE-cellulose; G.E.	(153)
Horse radish phosphomonoesterase	TEAE-cellulose; G.E.	(154)
Cysteinylglycinase	DEAE-cellulose; Dowex-2; S.W.D.	(155)
Rattlesnake venom; (phosphodiesterase, deoxyribonuclease, lecithinase, and L-amino acid oxidase)	DEAE-cellulose; G.E.	(156)
Glutamic-aspartic transaminase	CM-cellulose; S.W.D.	(157)
Pituitary protein hormones	DEAE-cellulose; S.W.D.	(158)
Horse radish peroxidase	Dowex-2, 8-10X; S.W.D.	(154)
Rattlesnake phosphodiesterase	Dowex-1, 2X; S.W.D.	(159, 160)
Serum trypsin inhibitor	Dowex-2; S.W.D.	(161)
Follicle stimulating hormone	Calcium phosphate; G.E.	(162)
Tyrosinase	DEAE-cellulose; S.W.D.	(163)
Carbamyl phosphate synthetase	DEAE-cellulose; cellulose-phosphate; G.E.	(164)
Rat liver supernatant	TEAE-cellulose; G.E.	(165)
Alder pollen allergens	DEAE-cellulose; SM-cellulose; S.W.D.	(166)
Enolase	SM-cellulose; specific adsorption with Mg^{++} form of adsorbant	(167)
Histones	CM-cellulose; G.E.	(168)
Histones	IRC-50; G.E.; S.B.D.	(169)
Prostatic acid phosphatase	Dowex-50 X2; S.W.D.	(170)
α-Glycoproteins	Amberlite XE-64; S.W.D.	(171)
Trypsinogen	IRC-50; S.B.D.	(172)
Gastricin	IRC-50; S.B.D.	(173)
Serum albumin	Dowex-2, 8-10X; S.W.D.	(174)
Red cell phosphatase	Dowex-2, 8-10X; S.W.D.	(154)
Kidney alkaline phosphatase	Calcium phosphate; G.E.	(175)
Pig liver cytochrome—556	Calcium phosphate; S.W.D.	(176)
DPNH dehydrogenase	Calcium phosphate; S.W.D.	(177)
Bovine γ-globulin	Two phase partition chromatography	(178)
Clupein	Alumina; S.B.D.	(179)
Guinea pig insulin	Silane treated Kieselguhr; reverse phase partition chromatography	(180)

* The following abbreviations are used: DEAE-cellulose for diethylaminoethyl cellulose; TEAE-cellulose for triethylaminoethyl cellulose; SM-cellulose for sulfoxymethyl cellulose; CM-cellulose for carboxymethyl cellulose; G.E. for gradient elution; S.W.D. for stepwise development; S.B.D. for starting buffer development.

ion-exchangers, introduced by Peterson & Sober (182), appear to be very versatile and have been extensively used.

Elution techniques are also illustrated by the examples of Table III. Boman (151) has listed these as (a) starting agent development, (b) gradient development, and (c) stepwise development. Starting agent development is defined as elution with the agent used for the column equilibration. This technique is particularly useful for examining the homogeneity of a protein, e.g., trypsinogen. Gradient development is defined as development with an agent whose composition is changed continuously from the initial solution toward one of higher eluting power. This method may be time-consuming but it takes advantage of the great changes in adsorption properties of proteins that occur with small changes in pH or ionic strength. An example is the resolution of the components of pancreatic juice. Stepwise development depends on the use of reagents differing from the one used to equilibrate the column. This technique may produce several zones with the same substance or one zone with many substances. Needless to say, all these techniques are empirical and no fixed rules can be formulated.

Despite some successes, methods involving greater selectivity and applicability to certain groups of proteins are needed; e.g., ion exchange chromatography of many globulins is impossible because of sparing solubility at the low ionic strength required for chromatography. Increased selectivity may be difficult to obtain, although knowledge of the protein in question may suggest a novel method as in the case of enolase. Malmström (167) adsorbed this metal-enzyme selectively on Mg^{++}-sulfoxymethylcellulose, advantage being taken of the high affinity of enolase for Mg^{++} ion as compared to the low affinity of impurities.

Zone electrophoresis.—The principles of zone electrophoresis were reviewed by Tiselius & Flodin (183) in 1953. Recent literature has been devoted to technical refinements and to applications to specific proteins and peptides.

Flodin & Porath (184) studied the electrophoretic behavior of peptides and proteins on vertical starch columns and have pointed out the advantages of this method, viz., quantitative results of high reproducibility and repetitive use of the same column. However, Raacke (185) has evaluated the electrokinetic changes in starch which might cause difficulties. Flodin & Kupke (186) note that starch is not an ideal supporting medium because of its instability, tendency to grow molds, ability to adsorb certain proteins, and its tendency to allow electroosmotic flow when used with polyvalent buffers. They suggest the use of cellulose powder or cotton modified by treatment with dry methanolic HCl. Porath has described apparatus which is suitable for analytical (187) and preparative work [(188); Gedin & Porath (189)].

Poulik & Smithies (190) have developed a two-dimensional electrophoresis system of high resolving power. A protein mixture is first developed in one direction on filter paper and then at right angles in starch

gel. More than 20 different protein components of serum were resolved in this system.

Countercurrent distribution.—The principles of this technique are well-known (191), and the high selectivity of the method is apparent from the reports of Craig and co-workers, who separated insulin from des-amido insulin (192) and sheep insulin from beef insulin (193). The major difficulty has been to find conditions which do not denature protein either at the interface between the phases or from the shaking involved in equilibration. Hausmann & Craig (194) have examined nearly 100 solvent systems to find two which appeared to be suitable for purifying human serum albumin. Acetyltryptophan and trichloroacetic acid were used as stabilizers. Although an ideal system was not found because of concentration effects of both albumin and trichloroacetic acid, Hausmann & Craig demonstrated that albumin is easily separated from other proteins although the albumins are not resolved. However, they mention new, but unpublished, systems capable of resolving both human and bovine serum albumin into several components.

King & Craig (195) describe systems which permit countercurrent distribution of lysozyme and ribonuclease. Upon comparison of the distribution patterns with column chromatography of fractions obtained, it appears that in addition to the main ribonuclease component, comprising 72 per cent of their original preparation, at least six minor components were present. However, enzymic studies were not made on these materials. The lysozyme preparation showed about 91 per cent of a major component, whereas activity determinations revealed no increase in activity of this component compared to the original enzyme. Attempts to find a system for distribution of chymotrypsinogen were unsuccessful.

Ellfolk (196) obtained electrophoretically homogeneous α-casein by using a three component system of collidine-ethanol-water. No attempt was made to prepare homogeneous β- and γ-casein by this procedure.

Dialysis.—Craig & King (197) have attempted to develop dialysis methods for protein fractionation. Examination of the rate of escape of various solutes through commercial membranes demonstrated that the 50 per cent escape times were a function of the molecular size of the solute. By use of a series of dialysis units analogous to a few stages of a countercurrent distribution system, it was shown that peptides obtained from a partial hydrolysate of tyrocidin-B, could be separated into groups of roughly the same molecular size.

Realizing that dialysis could be used to achieve better fractionation only if membrane selectivity could be enhanced, Craig, King & Stracher (198) have studied the escape rate of substances varying in size up to a molecular weight of 45,000 with a variety of membranes. In general, selectivity increases as the molecular size of a solute approaches the limit passed by a membrane. A membrane could be made more porous by placing it under hydrostatic pressure or less porous by acetylation. The influence of salt concentration, pH, binding with the membrane, and solute association-dis-

sociation on the rate of escape of proteins are described. This technique has been reviewed by Craig and co-workers (199).

Pierce & Carsten (200) have taken advantage of the membrane selectivity to determine the size of thyrotropin. A membrane of known porosity was stretched across the path of a migrating protein in a zone electrophoresis apparatus of starch gel. Depending on its size, the protein migrated through the membrane or around it. Thyrotropin would not go through a membrane which passed most proteins up to a molecular weight of 24,000 but would do so if the membrane was stretched. It was concluded that the hormone has a size of 26,000 to 30,000. A preliminary account of the behavior of other proteins ranging in molecular weight from 14,000 to 45,000 is also given.

STUDIES OF VARIOUS PROTEINS

Ribonuclease.—Earlier work on the structure and activity of this enzyme has been reviewed by Anfinsen & Redfield (201) and by Moore, Hirs & Stein (202). More recent information is found in the summaries of Hirs, Stein & Moore (101) and of Anfinsen (203, 204). Only a brief account of some of the more recent findings can be given here.

The Rockefeller group has described almost the complete sequence of amino acids in ribonuclease in a series of studies beginning with the amino acid composition (3) of the chromatographically homogeneous enzyme (205). Enzymic hydrolysates of the oxidized enzyme (56, 206) were prepared, and the resulting peptides were isolated by ion exchange column chromatography. Tryptic hydrolysis (207) yielded 13 principal peptides which accounted exactly for the 124 residues in the molecule. Eighteen peptides were obtained after chymotryptic hydrolysis (208), which accounted for more than the 124 residues in ribonuclease, but some of the fragments represented overlapping sequences. Hydrolysis with pepsin (209) yielded eight peptides which accounted for less than 60 per cent of the 124 amino acids. From the amino acid composition of these peptides a partial formula was deduced on the basis of the following assumptions: (*a*) ribonuclease is a single chain; (*b*) amino acid residues are linked by peptide bonds involving only α-amino and α-carboxyl groups; (*c*) peptides obtained from trypsin action contain lysine or arginine in the C-terminal position; and (*d*) the peptides obtained from chymotryptic digests contain C-terminal tyrosine or phenylalanine if one of these amino acids is in the peptide. Only one formula was found to accommodate all the information. To assign an exact location to each residue, the techniques of end group analysis and stepwise degradation are being used.

The location of the disulfide bridges in the molecule was accomplished by the same techniques employed by Sanger (9) for insulin. The location of two bridges was established by Spackman, Moore & Stein (210) and the others by Ryle & Anfinsen (211). Present information concerning the structure of ribonuclease is shown in Fig. 1; the position of residues shown parenthetically has not yet been described.
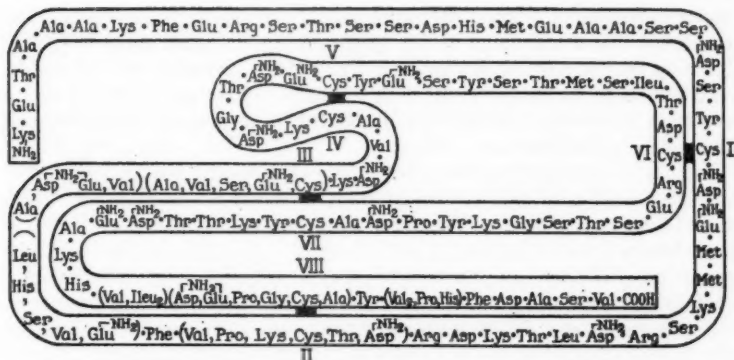
FIG. 1. A diagrammatic representation of a two-dimensional structure of ribonuclease. The half-cystine residues are numbered in the order in which they occur along the chain from the N-terminus of the molecule. The sequence of residues in parentheses has not been established. This representation can be considered as a working hypothesis of the structure of ribonuclease and is based on the degradative studies described in the text. The figure includes the information presented by Hirs, Stein & Moore (101). *(Diagram.—courtesy of Dr. S. Moore)*

In light of these studies, ribonuclease is being studied further in attempts to correlate its structure and activity. Earlier studies have been reviewed by Anfinsen & Redfield (201) and by Anfinsen (204).

Richards (212) has shown that subtilisin cleaves the alanyl-seryl bond between residues 20 and 21 from the N-terminal end of ribonuclease. The product was fully active after chromatographic purification, although, when the material was treated with trichloroacetic acid (TCA), neither the resulting insoluble fraction nor the TCA-soluble peptide exhibited enzymic activity. However, mixing of these two fractions resulted in a stoichiometric recombination and in regeneration of activity. Treatment of the 20-residue peptide with either carboxypeptidase, 8 M urea, or sodium periodate did not affect the recombination, whereas digestion with trypsin, or photo-oxidation, destroyed its ability to recombine to give a fully active enzyme. Treatment of the inactive TCA-insoluble protein with 8 M urea, trypsin, sodium periodate, or photo-oxidation resulted in loss of ability to recombine with the 20-residue peptide. Richards suggests that the peptide is bound to the protein by very strong noncovalent bonds which might help to maintain the molecule in its three-dimensional, active configuration.

Uziel, Stein & Moore (213) found that limited tryptic digestion of ribonuclease in the presence of 2 M guanidine resulted in hydrolysis of three to five peptide bonds. Chromatography of the product on Amberlite IRC-50 showed the emergence, after the native enzyme, of a new peak which was enzymically active and possessed the same amino acid composition and

molar extinction at 278 and 284 mμ as unmodified ribonuclease. The authors concluded that a maximum of five lysyl and arginyl bonds could be hydrolyzed by trypsin without producing a free peptide or amino acid. However, on the basis of Richards' results with subtilisin, it now appears necessary to ascertain whether trichloroacetic acid could aid in separating peptide material from the digested product.

It has been reported by Anfinsen (214) that limited pepsin digestion at pH 1.8 caused a parallel release of the C-terminal tetrapeptide and a decreased activity. However, Rogers & Kalnitsky (215) have used carboxypeptidase, which was not treated with diisopropylphosphorofluoridate, for degradation of ribonuclease. Such carboxypeptidase preparations are known to contain endopeptidases and residues are released which are not present at the C-terminal end of ribonuclease. Despite this, however, they reported that inactivation of ribonuclease was not as great as the amounts of some C-terminal amino acids liberated during the digestion. By comparing the kinds and number of residues released with the known amino acid sequences of ribonuclease they concluded that the active center of the enzyme is located near the C-terminal end of the molecule.

Chemical modifications of ribonuclease have been reported by several workers. Klee & Richards (216) used O-methylisourea for preparation of guanidinated ribonuclease. It appears, from this work as well as from other studies to be described below, that O-methylisourea is a useful reagent for guanidination of alkali-stable proteins. Fully guanidinated ribonuclease was shown, by its ultracentrifugal patterns and amino acid composition, to be homogeneous but it was enzymically inactive. When only nine of the possible ten lysine residues were converted to homoarginine, the product became fully active. It appears that one lysine residue of ribonuclease is not as reactive as the others, although when this residue reacts there is a loss of enzyme activity. No evidence was found for reaction of the single α-amino group of ribonuclease with O-methylisourea.

Brown et al. (217), have reported that guanidination yielded a chromatographically homogeneous product possessing about 10 per cent of the original ribonuclease activity. Because these workers were not certain whether nine or ten lysine residues were modified, their results would be consistent with those of Klee & Richards if approximately 90 per cent of the material was fully guanidinated and about 10 per cent had reacted only to the extent of nine lysine residues per mole. Treatment of ribonuclease with nitrous acid at pH 4 resulted in a deaminated product which was separated into two main components by chromatography. The properties of these two components were very similar and while each was enzymically active, no direct comparison with native enzyme was possible because the activity of the deaminated and the native enzyme differed at various ionic strengths. To determine groups responsible for the antigenicity of ribonuclease, these modified enzymes were tested in the ribonuclease-antiribonuclease system. The reactivity of the guanidinated enzyme with antibody

was about the same as that of native enzyme, whereas deaminated enzyme precipitated less antibody. Other immunological approaches to the study of ribonuclease are presented by Cinader & Pearce (218).

Taborsky (219) has phosphorylated ribonuclease by reaction with 1,3-diphosphoimidazole and obtained an active product which was chromatographically different from native enzyme. He has suggested that the phosphate was probably bound to two to five ε-amino groups of lysine. This would indicate that some ε-amino groups, unessential for activity as demonstrated by studies reported above, are also not essential even when the cationic charges of some ε-ammonium groups are replaced by negatively charged phosphate groups.

Josefsson (220, 221) has modified ribonuclease by exposing it to anhydrous formic acid. It had been previously shown for lysozyme (222) that anhydrous formic acid caused a reversible N,O-acyl migration at the hydroxyamino acid residues. Ribonuclease was inactivated by the formic acid treatment and reactivation was accomplished at alkaline pH. The reactivation closely paralleled the base consumption expected on the basis of the N,O-acylshift although maximal activity was not restored until exposure at a pH as high as 8.5 was achieved. Base consumption indicated that a minimum of 22 of the 25 hydroxyamino acids participated in an acyl shift. Although the inactive material differed from the native enzyme as judged by a higher cathodic electrophoretic mobility at pH 3.9, a higher viscosity, and a much higher sedimentation rate, each of these properties returned to normal on reactivation. This work suggests that all or a part of the primary structure around the many hydroxyamino acids is essential for ribonuclease activity, although no distinction can be made as to whether the acyl shift itself disrupts essential structures in the active site or disturbs the structure to such an extent that stabilization of the configuration at the active site is no longer possible.

Thus far, it has not been possible to implicate specific residues as partincipants in the active site of ribonuclease. It has been suggested that histidine (223), lysine (212), tyrosine (224, 225), and sulhydryl groups (226) all participate in some manner in maintaining the enzymic activity. [However, several investigators can find no evidence for SH groups in this enzyme (201, 204)]. Although some of these groups may play such a role, it is difficult to distinguish between the role they might play in maintaining the three-dimensional structure of the enzyme and that which would pertain to direct participation in the active site.

Trypsin.—Several approaches are being used to elucidate the relation between the structure of trypsin and its enzymic activity, e.g., (*a*) studies on zymogen activation; (*b*) kinetic studies with model compounds related to the enzyme or its substrates; (*c*) amino acid sequence analysis of the active site or sites; and (*d*) enzymic or chemical modification of the native enzyme. The first two approaches have been reviewed by Dixon, Neurath & Pechère (227). Analysis of the enzymically active region in trypsin has

been accomplished by labeling the enzyme with P^{32} organophosphates, and hydrolysis by enzymic or acid procedures, to yield P^{32}-labeled peptides. Dixon, Kauffman & Neurath (111) have obtained several peptides, one of which contains 15 residues in the sequence: Asp·Ser·CySO$_3$H·Glu·Gly· Gly·Asp·Ser(DIP)·Gly·Pro·Val·CySO$_3$H·Ser·Gly·Lys, where DIP represents the diisopropylphosphoryl moiety. A discussion of the significance of this sequence with respect to the mechanism of action of trypsin will be presented below in the discussion of chymotrypsin.

Rovery, Fabre & Desnuelle (228) have shown that the first nine amino acids in the N-terminal region of trypsinogen possess the sequence Val· (Asp)$_4$·Lys·Ileu·Val·Gly. Earlier work (229, 230) had demonstrated that the terminal hexapeptide is liberated during activation by trypsin. Gabeloteau & Desnuelle (231) have examined peptides isolated from autolysates of trypsin; whereas one of these corresponded to the N-terminal sequence, several others could not be assigned to any definite region in the molecule.

Recent studies on S-sulfotrypsinogen have confirmed that this protein, like trypsinogen itself, does not yield stoichimetrically significant amounts of amino acids on treatment with carboxypeptidase A or B (68). Trypsin, on the other hand, has been shown to be attacked by carboxypeptidase B to yield several equivalents of lysine per mole (136).

Sorm and his colleagues (26, 28, 31) examined many peptides obtained from partial acid hydrolysates of trypsinogen and chymotrypsinogen and were able to show that both proteins contain dipeptides of the same structure. Peptides with the following sequences were found: His·CySO$_3$H; Thr·CySO$_3$H; Phe·CySO$_3$H; Ser·Arg; Val·Arg; Ala·His; Ser·Lys; Thr·Lys; and Lys·Leu. Several other peptides whose sequences were unknown seemed to be identical or very similar. Although it is apparent that this kind of work will not provide enough information for logical sequence analysis, it will elucidate regularities which might occur in the arrangement of amino acids within protein chains and, in particular, in those proteins which have a related biological function or origin.

Several reports have described chemical or enzymic modifications of trypsin. Nord and co-workers studied the acylation of trypsin by various acid anhydrides. Determination of the specificity of the acetylation in aqueous media (232, 233) showed that amino groups reacted at low pH. In more alkaline buffers complete acetylation of amino groups occurred with some reaction of phenolic hydroxyls. In each case the acetylated trypsins were enzymically active. However, acetylation of trypsin in dimethylsulfoxide or formamide resulted in inactivation. It would appear that a new specificity of reaction toward certain reactive groups occurs in organic solvents that is not found in aqueous systems. Succinyl trypsin is the most stable acyltrypsin so far prepared (234): it exhibits a heat stability comparable to that of trypsin at acid pH as well as showing a greater heat stability than trypsin at alkaline pH. More significantly, succinyl trypsin is reported to undergo self-digestion without loss in activity. However, the results

of Wootton & Hess (235), as well as those of Liener (236), suggest that acetylated trypsin does not produce active fragments on autolysis. Instead, these workers concluded that autolysis of acetyl trypsin involves hydrolysis of denatured enzyme produced during acetylation, thus allowing dialysable material to form without loss of activity. After prolonged autolysis, the residual active enzyme contained the identical number of free amino groups per mg. acetyltrypsin N as before autolysis.

Viswanatha, Wong & Liener (237, 238) report that trypsinogen, with 88 per cent of its amino groups acetylated, is digested by pepsin to yield a fragment which manifests trypsin activity toward benzoylarginine ethyl ester. Purification is stated to yield a product with about seven times the specific activity of trypsin. The substance is reported to be homogeneous in the ultracentrifuge and to be about one-fourth the size of native trypsin.

Confirmation of the observation of Bresler *et al.* (239) and Chernikov (240), was made by Hess & Wainfon (241), who report that partial autolysates of trypsin possess proteolytically active material which diffuses through a semipermeable membrane at a faster rate than native trypsin.

Chymotrypsin.—Studies of chymotrypsin have followed many of the same lines as those of trypsin. Because recent reviews by Neurath and colleagues (227, 242) have considered these in some detail, only newer studies will be discussed.

The amino acid composition of α-chymotrypsinogen has been reported by Wilcox, Cohen & Tan (7). Studies with chymotrypsinogen, as well as with the neochymotrypsinogens, have provided a knowledge of the end groups as well as the peptide products liberated during activation (242, 243, 244).

Meedom (245) examined the products of performic acid oxidation of chymotrypsinogen and chymotrypsin. α-Chymotrypsin gave three major fragments: one representing the bulk of the molecule, a second peptide containing about 50 residues, and a third possessing 13 residues in the sequence (246) $CySO_3H \cdot Gly \cdot Val \cdot (Ala,Pro) \cdot Ileu \cdot Val \cdot Pro \cdot GluNH_2 \cdot Leu \cdot Ser \cdot Gly \cdot Leu$. Hartley (247) has examined the longest peptide and found that this material contains both histidines of chymotrypsin as well as the sequences around the active site. Although this peptide contains 160 residues, a partial sequence of 15 amino acids containing two arginine residues has been found.

As mentioned above, the C-terminal sequence of S-sulfochymotrypsinogen has been determined by Neurath, Dixon & Pechère (68), using rate studies after treatment with carboxypeptidase A, as: $Try \cdot (Ser,Ala) \cdot Val \cdot Thr \cdot Leu \cdot Ala \cdot Asp(NH_2)COOH$.

Other reported structural studies are primarily concerned with the sequence labeled by treatment of chymotrypsin with radioactive organophosphates, such as diisopropyl phosphorofluoridate (DFP). Since such investigations have also been performed with other organophosphate-sensitive enzymes, the following discussion will not be limited to chymotrypsin.

Jansen and co-workers (248) first showed that reaction of chymotrypsin with organophosphates is stoichiometric, one mole of inhibitor reacting with one mole of the enzyme to produce irreversible inactivation. From this, it is generally believed that reaction occurs at the active site of the enzyme. Consequently, the peptide sequence which bears a firmly bound organophosphoryl moiety probably represents at least a part of the active site. These considerations are applicable not only to chymotrypsin, but to trypsin (249, 250), phosphoglucomutase (251), thrombin (252, 253), cholinesterases (254), and ali-esterases (254). Each of these enzymes has been treated in this manner and the resulting labeled peptides isolated.

Schaffer *et al.* (255), were the first to isolate and characterize labeled peptides from chymotrypsin. They found the sequence Gly·Asp·Ser(DIP) ·Gly·Glu·Ala, with the DIP (diisopropylphosphoryl) label on the serine. This sequence has been confirmed by Turba & Gundlach (256). Oosterbaan *et al.* (257, 258) have also isolated a labeled peptide, which is reported to have the sequence Gly·Asp·Ser(DIP)·Gly·Gly·Pro·Leu. Only four residues of the seven residue sequence agree with that reported by Schaffer *et al.* and by Turba & Gundlach. These differences remain to be explained.

Oosterbaan & Van Adrichem (259) have also examined peptides obtained by peptic digestion of acetyl chymotrypsin, prepared by treatment with p-nitrophenylacetate labeled with C^{14} in the carboxyl group of acetate. Five labeled peptides were isolated from the digests and, although no sequences were determined, the composition of the peptides was compatible with the sequence Gly·Asp·Ser·Gly·Gly·Pro·Leu, where the serine residue bears the acetyl label.

The structure of the 15-residue labeled peptide obtained by Dixon *et al.* (see above, p. 116) from trypsin is consistent with the peptides isolated from partial acid hydrolysates of trypsin which had been labeled with isopropylmethylphosphorofluoridate-P^{32}. Schaffer *et al.* (260) showed these peptides to be Ser(PO_3CH_3); Asp·Ser(PO_3CH_3)·Gly; and Asp·Ser (PO_3CH_3)·Gly. The composition of the P^{32}-labeled peptides isolated from enzymic hydrolysates of DIP^{32}-trypsin by Oosterbaan, Jansz & Cohen (261) is also in accord with the sequence of Dixon *et al.* Their peptide contained Gly$_{2-3}$,Asp,Ser, Pro,Val, and P^{32} in unknown sequence.

The isolation of DIP peptides from partially digested, labeled thrombin, has been reported by Gladner and his colleagues (262, 263); they obtained a peptide which they believe corresponds to the sequence Gly·Asp·Ser (DIP)·Gly. Here again, studies are needed to determine this sequence.

The behavior of phosphoglucomutase is not quite the same as that of the enzymes just discussed. Larger amounts of organophosphate are required for complete inhibition of phosphoglucomutase (251). The peptides containing the active site were not obtained by reaction with DFP but were labeled with P^{32} phosphate, which is known to bind covalently with the enzyme (264). Koshland & Erwin (265) report that all the labeled peptides contain aspartic acid, serine, glycine, and glutamic acid and, although no

Sequences were determined, the authors conclude that phosphoglucomutase contains the same amino acid sequence in its active site as chymotrypsin.

Oosterbaan and co-workers (254, 261) present results which suggest that the structure around the P^{32} label in liver DIP ali-esterase, serum DIP pseudocholinesterase, red cell DIP cholinesterase, and red cell DIP ali-esterase might be the same as those established for trypsin and chymotrypsin. Determination of the exact nature of these peptides will require more rigorous analysis before they can be included with these enzymes.

Table IV presents a summary of the sequences believed to occur in the active site of those enzymes which are inhibited by DFP, as well as the

TABLE IV

LABELED PEPTIDES FROM SEVERAL ORGANOPHOSPHATE SENSITIVE ENZYMES

Enzyme	Sequence	Reference
Chymotrypsin	Gly·Asp·Ser(DIP)·Gly·Glu·Ala	(225)
Chymotrypsin	Gly·Asp·Ser(DIP)·Gly·Glu·Ala	(256)
Chymotrypsin	Gly,Asp,Ser(acetyl),Gly,Gly,Pro,Leu	(257, 258)
Trypsin	AspNH$_2$·Ser·CySO$_2$H·Glu·Gly·Gly·Asp·Ser	
	(DIP)·Gly·Pro·Val·CySO$_2$H·Ser·Gly·Lys	(111)
Trypsin	Asp·Ser(PO$_3$CH$_3$)·Gly	(260)
Thrombin	(Gly,Asp,Ser(DIP),Gly)	(262)
Phosphoglucomutase	(Asp,Ser(PO$_4$),Gly,Glu,Ala,Val)	(265)

compositions of labeled peptides in which sequences have not been established. It is important to note that the first three sequences presented for chymotrypsin and the two sequences for trypsin are the only ones in which sequences have been determined. It is also worth re-emphasizing that there is unexplained disagreement in the results for chymotrypsin.

The presumed similarity of the sequences around the labeled serine of phosphoglucomutase and chymotrypsin has led Koshland & Erwin (265) to suggest a common bond-breaking mechanism for these enzymes, i.e., the common sequence around the serine is involved in making an otherwise inert hydroxyl group of serine reactive. No suggestion is given, however, to explain what properties of the sequence Gly·Asp·Ser·Gly confer this reactivity on the serine hydroxyl group. Furthermore, it is postulated that the different specificities of these enzymes should be reflected in another portion of the enzyme which is adjacent to the active site although in perhaps a separate coil of the molecule. These ideas are consistent with the views of Gutfreund (266), Cunningham (267), and Dixon, Neurath & Pechère (227), who have proposed that the catalytic structure in the active site of chymotrypsin involves a histidyl-seryl interaction between an un-ionized imidazole of histidine and the hydroxyl group of serine. The serine group was primarily invoked by these workers to explain its labeling after reaction with

DFP. It is important to point out, however, that some workers feel that the DIP labeling of serine represents a secondary point of attachment of the phosphate (268).

An explanation for the reactivity of the serine side chain has been offered by Porter, Rydon & Schofield (269). They propose that the serine hydroxyl and nitrogen in the active site are part of a Δ^2-oxazoline. Although no evidence for the presence of the oxazoline structure in native proteins at neutral pH values has been found since Bergmann, Brand & Weinmann (270) first suggested this possibility many years ago, Porter and his colleagues have examined the reactivity of three different synthetic oxazolines. They found that two different Δ^2-oxazolines could react at 37° in aqueous $NaHCO_3$ with DFP to give a product which on acid hydrolysis yielded O-phosphoethanolamine. Rydon (271) has proposed a reaction mechanism for the DFP-sensitive enzymes which depends upon the reactivity of the serine as a Δ^2-oxazoline and also upon the β-carboxyl group of the aspartic acid residue which is ubiquitously present, immediately N-terminal to the reactive serine (see Table IV). Although this reasonably explains the reactivity of the serine, no clear suggestion is offered to explain the role of the histidine residue thought to be present in the active site.

Westheimer (272) has attempted to describe in detail the orientation of the active site of chymotrypsin by assuming that the folding of the hypothetical sequence unique to this enzyme, as well as to trypsin, is an α-helix which serves to bring the histidine and serine into the close proximity required of the seryl-histidyl bond suggested to be operative in the active site. Dixon *et al.* (111) have pointed out that this hypothesis is not consistent with the determined sequence around the labeled seryl residue of trypsin in that no histidine is present in the 15-residue peptide studied nor even in the largest labeled peptide isolated (55 residues). They also indicate that the proline and half-cystine residues present in the 15-residue peptide, probably preclude the formation of an α-helix in this region of trypsin.

Pepsin—Van Vunakis & Herriott (273) have examined one of the peptides liberated from pepsinogen after activation and have shown it to have a molecular weight of 3000 to 3200. It appears, however, that at least five other peptides are also liberated. On the basis of end group determinations it appears that pepsin does not occupy the N-terminal position in its zymogen, pepsinogen. A summary of these studies has been presented by Dixon, Neurath & Pechère (227).

Perlmann (274) reported that pepsin can autolyze to yield dialysable products which are active. This material of low molecular weight had a higher relative activity towards synthetic substrates than towards hemoglobin. A further study by Perlmann & Mycek (275) has shown that urea-treated pepsin slowly autolyzes to produce new protein or peptide material. Whereas the major component of this mixture had the same mobility as pepsin, sedimentation studies and amino acid analysis revealed that it was somewhat different although apparently not small enough to pass a semi-

permeable membrane. This material was 40 to 50 per cent more active than the parent pepsin, although specificity differences, such as were reported for the dialysable fragment, were not observed.

Other work by Perlmann (276) has considered the nature of the phosphorus in pepsin and pepsinogen. Complete dephosphorylation of pepsin and pepsinogen occurs after treatment with either intestinal phosphatase at pH 8.9 or potato phosphatase at pH 5.6. The dephosphorylated pepsin was fully active and, after activation of phosphorus-free pepsinogen with HCl, the resulting product was active. Furthermore, both dephosphorylated substances were electrophoretically different from the parent molecules, exhibiting a decreased cathodic mobility. Studies of the nature of the phosphorus linkage by enzymic as well as physical methods, led to the conclusion that the single mole of phosphate in pepsin is in diester linkage. Since pepsin is a protein with a single peptide chain, Perlmann suggests that the phosphate serves to cross-link a portion of the chain into a cyclic loop.

Lysozyme.—Earlier studies by Schroeder (277) and Thompson (278) described certain of the amino acid sequences in the primary structure of lysozyme. Fromageot and his associates (280, 281, 282) and Jollès & Thaureaux (283, 284) have established some of the sequences around specific amino acids in the molecule. More recently, the peptides isolated from tryptic (57) and peptic (142) digests have allowed these workers to assign a partial structure to lysozyme which accounts for more than one-half of the composition of the enzyme. The recent review by Jollès, Jollès-Thaureaux & Fromageot (279) summarizes the progress made to date.

Geschwind & Li (285) have reported that complete guanidination of lysozyme occurs without alteration of the lytic activity. Although no physical studies were presented, dinitrophenylation studies revealed a single end group of homoarginine which would be formed from the N-terminal lysine of the enzyme. The guanidinated derivative was not inactivated by acetylation, whereas acetylation of native lysozyme resulted in a material with only one-third of its original activity. This points to the necessity of maintaining cationic side chains in lysozyme regardless of whether they are ϵ-ammonium or ϵ-guanidinum functions.

Josefsson & Edman (222) have studied the effects of anhydrous formic acid on lysozyme. When lysozyme is exposed to formic acid at 25° there is a progressive loss of its enzymic activity, complete inactivation occurring in 16 hr. The inactivated product could be reactivated by incubation at pH 7.5 to 8.0 for several hours. They believe that the modification involves a reversible N-O peptidyl shift (acyl shift) as in the case of ribonuclease discussed above. Although more evidence for the proposed shift is desirable (e.g., formation of derivatives of the new amino group produced after treatment with formic acid) before the interpretation of the authors is accepted, it is apparent that the studies of ribonuclease and lysozyme represent an important contribution.

The action of tyrosinase on lysozyme has been studied by Yasunabu &

Wilcox (286). Although no alteration of tyrosine occurred, it is noteworthy that they showed that α-lactalbumin, which is similar to lysozyme in many of its chemical features, was markedly altered by the tyrosinase.

Papain.—The review by Kimmel & Smith (287) provides a summary of the chemical, physical and enzymic properties of this enzyme. Other studies have been reviewed by Smith (288) and by Smith, Hill & Kimmel (289).

The primary structure is being studied in some detail by Kimmel, Smith and colleagues (58, 290, 291), who have approached the problem in much the same manner as that described above for ribonuclease and lysozyme. Tryptic digestion (291) of performic acid oxidized papain and subsequent ion exchange chromatography resulted in the identification of at least 18 different peptides, some of which have been partially characterized. Some progress has been made in correlating the sequences of some of these peptides with peptides obtained from chymotryptic digestion of oxidized papain. However, these studies have been hampered by the presence of a resistant, insoluble "core" after digestion with either trypsin or chymotrypsin. On the basis of studies in which papain was labeled with C^{14} iodoacetamide (290), almost all of the radioactivity was found in the insoluble core, indicating that the single reactive thiol of papain is in this material. Isolation of a peptide containing this label should provide information concerning this part of the active site.

Finkle & Smith (292) have examined the thiol groups of papain. On the basis of the reactivity of papain with mercury, organic mercurials, and iodoacetamide, they showed that the specific activity of the enzyme involves only one of the six thiol groups in the molecule. Direct titration of this thiol group was possible by a spectrophotometric analysis of its reaction with iodoacetamide as well as by the Boyer technique of following spectrophotometrically, the reaction with *p*-chloromercuribenzoate. These titrations indicated that the specific activity of different preparations of papain was directly proportional to the amount of a single, reactive thiol group. However, under different conditions all six thiol groups could be made to react with *p*-chloromercuribenzoate which suggests that cystine itself is absent from papain and that six moles of cysteine are present.

Although an analysis of the kinetic behavior of papain does not fall within the scope of this review, it should be mentioned that kinetic data point to the direct participation of an essential thiol group in the k_1 step of the classical Michaelis-Menten formulation (293, 294, 295). In addition, it appears that an ionized carboxyl group is also intimately associated with the thiol group during this catalytic step.

Additional experiments on mercuripapain, degraded at its N-terminal region by leucine aminopeptidase, have been reported by Hill & Smith and their colleagues (296, 297). The extent of degradation in 24 hr. was found to be proportional to the ratio of aminopeptidase to the substrate (mercuripapain). After degradation, the enzyme could be fully reactivated by removal of the mercury. Furthermore, the degraded material showed an en-

tirely different pattern of amino end groups as determined by the dinitro-phenyl procedure. Amino acid analysis of the chromatographically separated, degraded mercuripapain shows that this material is different from intact mercuripapain only in those amino acids removed by the aminopeptidase (297). The sum of the amino acids liberated by aminopeptidase and those in the residual, degraded enzyme is in agreement with the composition of native papain. More recent, unpublished studies have demonstrated that extensively degraded mercuripapain could be purified by repeated chromatography on Amberlite IRC-50. The product appeared to be homogeneous, yielding a symmetrical elution curve in which activity and ninhydrin color coincide. On the basis of amino acid analyses, a size of approximately 75 to 80 residues was calculated. It is interesting that chromatographic analysis of an autolysate of papain (292) revealed the presence of active material which differed from intact papain. This result indicates that papain can autolyze much in the same manner as trypsin and pepsin to produce active fragments smaller than the native enzyme.

Hill *et al.* (297) have chemically modified mercuripapain with both nitrous acid and O-methylisourea. Guanidination with the latter reagent results in conversion of at least seven of the eight lysine residues in the molecule to homoarginine. This treatment does not markedly alter the crystallizability, specific activity, or substrate specificity. However, the electrophoretic mobility and chromatographic behavior are modified as is to be expected from the greater basicity of the guanidinium groups. On the other hand, treatment with nitrous acid irreversibly inactivates mercuripapain, whereas under the same reaction conditions only slight inactivation of the guanidinated enzyme occurs. These studies indicate the essential nature of basic side chains in some of the positions occupied by lysine, whether they are ε-ammonium or ε-guanidinium groups, as in the case of lysozyme (285) mentioned above.

A synthesis of presently available information on the activity and active site of papain has been presented by Smith (298), who has proposed a mechanism of action for this enzyme. On the basis of the chemical and kinetic behavior of the enzyme, it is assumed that a free thiol group is not present in the active enzyme but that a "high energy" bond of the type of a thiol ester involving an un-ionized SH group and a carboxylate ion is present. Furthermore, the thiol ester is formed and maintained through the energy derived from the favorable, folded configuration of the native enzyme. On the basis of these assumptions, a reaction mechanism is proposed which is compatible with and explains the catalytic properties of papain. This paper also discusses the possible role of bonds of the "high energy" type both in enzyme action and in the maintenance of three-dimensional protein structure.

Insulin.—Elucidation of the primary structure of this hormone by Sanger (9) has provided the basic methods for establishing the primary structure of all other proteins and peptides whose sequences have been studied more

recently. This work will not be reviewed here, but some experiments should be considered which attempt to relate the structure of insulin to its hormonal activity.

Nichol & Smith (299) have shown that tryptic digestion of intact insulin, as in the case of the oxidized B-chain (9), results in the cleavage of the arginyl-glycyl bond between residues 22 and 23 and the lysyl-asparaginyl bond between residues 29 and 30 in the B-chain. The residual insulin molecule was only about 15 per cent as active as the native hormone, whereas the heptapeptide (residues B 23 to 29) was completely inactive.

In order to gain insight into the role of the N-terminal sequence of insulin, Smith, Hill & Borman (300) digested insulin with leucine aminopeptidase. It is significant that only Zn-free insulin is susceptible to proteolytic attack by this enzyme and is considerably more susceptible to trypsin than is crystalline zinc-insulin (299). On the basis of the amino acids released from insulin by aminopeptidase and from assays of the residual insulin core, it was possible to show that the first six residues of the B-chain do not appear to be essential for hormone activity. However, the loss in activity was apparently attributable to hydrolysis of the A-chain in its immediate N-terminal region or to the breakdown of the B-chain past the first six residues.

Evans & Saroff (301) have prepared a physiologically active, guanidinated derivative of insulin, by treatment with O-methylisourea. The material crystallized readily and possessed sedimentation and solubility properties only slightly different from native insulin. Only a single homoarginine residue was found, as is to be expected from the presence of one lysine residue in the protein. However, unlike the behavior of chymotrypsinogen and ribonuclease, approximately one-half of the terminal α-amino group of glycine was guanidinated as was about 10 per cent of the N-terminal phenylalanine. These authors identified the α-guanido acids by use of the sensitive acetyl benzoyl reagent (302).

Koltun (303) has reported the reaction of p-carboxyphenyldiazonium sulfate with insulin. Aside from a different physical behavior, the p-carboxyphenylazo derivative contains more azo compound than expected from the tyrosine and histidine content, residues which were thought to react specifically with diazo compounds. This result is consistent with the results of Howard & Wild (304), who have carefully determined the specificity of diazotization of proteins.

Serum albumins.—The wide use of albumins as model proteins indicates that further structural information is needed. A general discussion of the structure of albumin has been given by Edsall (305). Chemical or enzymic methods of end group analysis have revealed the presence of one N-terminal aspartic acid (306, 307, 308) and one C-terminal alanine (bovine albumin) or leucine (human albumin) per mole of protein (117, 121, 309). These results suggest that the albumins are single polypeptide chains. This is supported by the work of McDuffie & Hunter (310), who have reported

that reduction of the disulfide bonds in albumin, followed by alkylation of the free thiol groups, yields a derivative which has the same molecular weight as the native protein. On the other hand, Reichmann & Colvin (59) claim that performic acid oxidation yields a derivative which has a lower molecular weight although it possesses no new end groups. Titani *et al.* (311) report finding N-terminal cysteic acid in oxidized equine serum albumin. Thompson (312), on critical re-examination of the end groups of oxidized bovine and equine albumins, could not confirm these results. Thus, on the basis of chemical evidence it appears that albumin is a single polypeptide chain of molecular weight close to 69,000. Alteration of this concept must await a clearer understanding of what has occurred during oxidation with performic acid.

Šorm (26) and his co-workers have examined the arginine peptides obtained from partial acid hydrolysates of the serum albumins from five different species. Although no sequence information was obtained, it was of interest that the ox and sheep peptides contained high levels of threonine compared to those derived from man, horse, and duck, whereas the duck peptides were low in tyrosine. These differences undoubtedly reflect the kinds of species variation in amino acid sequence around similar parts of the albumin molecule. Species differences in insulin (313), cytochrome-*c* (see the next section), and several protein and peptide hormones (314) have been described elsewhere.

Porter (315, 316) has obtained a fragment of bovine serum albumin which, although about one-fifth the size of the protein, still possesses ability to combine with specific antibody. This fragment was obtained by digestion of the albumin with chymotrypsin; the fragment was separated from the digestion products by allowing it to diffuse through a dialysis membrane. Zone electrophoretic purification yielded a material of about 12,000 M which could combine with rabbit antialbumin and which contained a single N-terminal phenylalanine residue. Furthermore, the active fragment could provoke anaphylactic shock in a guinea pig which had been passively sensitized with antialbumin. Although the groupings which are responsible for maintaining the antigenicity of the fragment are unknown, heat lability suggests that an intact three-dimensional configuration is needed for activity.

Cytochrome-c.—The porphyrin is attached to the protein through thioether linkages to the sulfur of cysteine residues (317 to 321). Tuppy (322) and his colleagues have determined the amino acid sequences of the part of the protein which bears the prosthetic group in various species. These sequences, given in Table V, show interesting similarities as well as differences. The presence of a basic residue adjacent to one cysteine and the common sequence, His·Thr·Val·Glu, next to the other cysteine residue are especially noteworthy. The details of the sequence analysis of these peptides have been reviewed (323).

The influence of the protein moiety on the electron transport function of cytochrome-*c* has been approached by several workers through chemical

TABLE V

SPECIES DIFFERENCES IN CYTOCHROME-C (322)

Species	Sequence*
Beef	Val·GluNH₂·Lys·CyS·Ala·GluNH₂·CyS·His·Thr·Val·Glu·Lys
Horse	···· Lys·CyS·Ala·Glu·NH₂·CyS·His·Thr·Val·Glu·Lys
Pig	···· Lys·CyS·Ala·GluNH₂·CyS·His·Thr·Val·Glu·Lys
Salmon	Val·GluNH₂·Lys·CyS·Ala·GluNH₂·CyS·His·Thr·Val·Glu
Chicken	Val·GluNH₂·Lys·CyS·Ser·GluNH₂·CyS·His·Thr·Val·Glu
Silk moth	Val·GluNH₂·Arg·CyS·Ala·GluNH₂·CyS·His·Thr·Val·Glu
Yeast	Phe·Lys·Thr,··Arg·CyS·Glu·Leu,···CyS·His·Thr·Val·Glu

* The sulfur of the CyS residues is bound by thio-ether bonds to the side chain of the porphyrin.

and enzymic modification of the native protein. Minakami and his colleagues (324, 325) have acetylated and guanidinated cytochrome-c. Material in which 90 per cent of the amino groups were acetylated appeared to be homogeneous by paper electrophoresis and had a cathodic mobility at pH 8.4, whereas the native enzyme had an anodic mobility at this pH. In addition, preparations in which more than 40 per cent of the amino groups had been acetylated could not be adsorbed to Amberlite IRC-50 under conditions where the native enzyme was adsorbed. Although no change was found in the absorption spectra of either oxidized or reduced cytochrome-c during acetylation, the enzymic reactivity was affected. The acetylated protein was readily auto-oxidizable, although it did not bind carbon monoxide. No stimulation of succinate oxidation was found when the modified material was added to a succinoxidase system. However, it was possible to oxidize and reduce the modified enzyme enzymically, although at very slow rates. In addition, ascorbic acid oxidase and hydroxylamine reductase activities were absent from the modified enzyme. The guanidinated enzyme, however, appeared to have the same biological activity in the succinoxidase system, the same absorption spectrum in the reduced state, and the same nonreactivity toward carbon monoxide as the unmodified enzyme. These results indicate the necessity of cationic groupings at certain positions whether they be lysine or homoarginine residues.

Nozaki *et al.* (326) report that the reduced form of cytochrome-c is less susceptible to a bacterial proteinase than the oxidized form. This suggests that reduction of cytochrome-c not only leads to a chemical change in the iron-porphyrin but also to a change in the configuration of the protein.

Catalase.—Anan (327, 328) has shown that both tryptic and peptic digestion of catalase gradually destroys its catalatic activity, whereas its peroxidatic activity is somewhat enhanced. Although both types of digestion produce mixtures of degraded catalase, partial purification of the peptic

digested material by chemical methods yielded a protein which was about two-thirds the size of native catalase. Whereas these degraded preparations might represent mixtures of varying activities, it is interesting that the purified material exhibited peroxidatic guaiacol oxidation, the oxidative Nadi reaction, and could aerobically oxidize L-ascorbic acid; all of the activities were inhibited by cyanide, azide, or fluoride. These results are similar to those with cytochrome-c, where a purified fragment from peptic digestion was shown to be auto-oxidizable and to possess ascorbic acid oxidase activity, although devoid of its electron transport function (321).

Enolase.—The availability of gram quantities of a highly purified, crystalline enolase of yeast (167) has allowed certain structural studies to be made with this metalloenzyme. Malmström, Kimmel & Smith (329) determined its amino acid composition. They found a single alanine residue at the N-terminus, in the sequence Ala·Glu·(Val,Lys). This indicates that it is a single polypeptide chain containing approximately 600 residues. Enolase proves to be among the few proteins completely devoid of both cysteine and cystine, methionine accounting for its total sulfur content.

Malmström (330) has studied the action of trypsin, carboxypeptidase, and leucine aminopeptidase on enolase. Tryptic digestion caused a rapid loss of activity. Both aminopeptidase and carboxypeptidase could remove many amino acids from enolase from either end of the single chain without altering its activity. At an enolase to peptidase molar ratio of 30 : 1 for the digestions, it was estimated by the ninhydrin method that carboxypeptidase could remove 80 amino acid residues from the C-terminal end of the molecule in 22.5 hr., whereas aminopeptidase could remove about 90 residues from the N-terminus in the same reaction time. Although these results indicate that the active site cannot lie at the extreme terminal regions of enolase, it is of interest that digestion of the molecule with one peptidase followed by the other, caused an irreversible inactivation. It would thus seem that the active site is stabilized in its required configuration by at least a part of the extreme ends of the molecule.

Phosphoproteins.—The status of the phosphoproteins was reviewed by Perlmann (331) in 1955. Alpha- and beta-caseins have received particular attention. Hipp, Groves & McMeekin (332) have obtained phosphopeptides from partial acid hydrolysates of α-casein. Chromatographic separation of the phosphopeptides from the hydrolysates resulted in the unequivocal identification of phosphoserylglutamic acid, phosphoserylalanine, and phosphoserylphosphoserine. Peterson, Nauman & McMeekin (333) isolated a much larger phosphopeptide from tryptic digests of β-casein. This peptide, which was electrophoretically homogeneous, was shown by two different methods to have a minimum molecular weight of 3111 and 4100, respectively. The empirical residue formula was shown to be NH_2-Arg· (Asp,Glu,$(NH_2)_3$, Gly,Val$_2$, Leu$_3$,Ileu$_2$,Ser$_4$,Thr,Pro,$(PO_4)_5$,Arg-COOH. Arginine was shown to be at the N- and C-terminal positions. Although these authors have not determined the positions of the phosphates, the composition found does not

support the conclusions of Perlmann (331), who indicated that all the phosphate in β-casein is in diester linkage. It is clear that the five hydroxyl groups of serine and threonine in the peptide are not sufficient to form diester linkages with the five phosphate groups. The authors have considered that diester linkages might have been broken by trypsin, but this possibility was dismissed after it was demonstrated that trypsin had no diesterase action on model substrates.

Hofman (334) has studied the action of a phosphoprotein phosphatase and prostate phosphomonoesterase on α- and β-caseins, as well as on several model substrates. His results are not in accord with those previously reported by Perlmann (331).

Hemoglobin.—Only certain structural studies of this protein will be considered here; more detailed accounts can be found in the review by Itano (335) and in the recently published *Conference on Hemoglobin* (85).

Of great importance to future structural studies are methods for the purification of crystalline human hemoglobin prepared by a procedure similar to that of Drabkin (336). Kunkel & Wallenius (337) have separated pure carboxyhemoglobins by zone electrophoresis on starch. These hemoglobins have been analyzed by Stein *et al.* (8), who showed that the major hemoglobin component contained no isoleucine, unlike many preparations which had been regarded as pure. Although the absence of isoleucine from normal hemoglobin can be used as a criterion of its purity, these authors suggest that other criteria such as sulfhydryl content should also be considered. [It is noteworthy that Brand & Grantham (338) had reported in 1946 that human hemoglobin lacks isoleucine.] Allen, Schroeder & Balog (339) have come to similar conclusions. Chromatography of both crystallized and uncrystallized human hemoglobin on Amberlite IRC-50 revealed the presence of three minor heme proteins and a nonheme protein which differ in isoleucine content. The main peak, which comprises 90 per cent of the hemoglobin, lacks isoleucine.

A discussion of the sulfhydryl groups of human hemoglobin has been given above. The earliest study of the N-terminal residues by Porter & Sanger (340) indicated five valyl residues per molecule, and subsequent investigations by Havinga (341), Masri & Singer (342), Huisman & Drinkwaard (343), and Brown (344) have supported this value. A recent study by Rhinesmith, Schroeder & Pauling (345, 346) indicates that there are four residues per mole. Careful examination of the hydrolysis of DNP-globin showed that 90 per cent of two residues per molecule was released as DNP-valyl-leucine in the first few minutes of hydrolysis with no significant amount of DNP-valine released. Thereafter, DNP-valine increased, but at a much slower rate than it was released from DNP-valyl-leucine. Thus it appears that free DNP-valine was being released from the other half of the hemoglobin molecule. Calculation of the first order rate constants for the release of DNP-valine from the two sources allows estimation of the total amount of DNP-valine which can be potentially released. This amounts to

exactly four residues per mole of hemoglobin. Aside from establishing the
exact number of end groups, these results demonstrate the presence of two
different kinds of chains in the molecule, a result compatible with the x-ray
studies of Perutz (347) and co-workers and the results of Ingram (348)
which will be discussed below.

Some of the amino acid sequences in hemoglobin have been established
by Ingram (349 to 352), who has examined the peptides resulting from the
tryptic digests of hemoglobin A as well as from several abnormal hemo-
globins associated with anemias (335). Tryptic digestion (353) yielded a
soluble peptide fraction, rich in arginine and lysine, and an insoluble trypsin-

TABLE VI

CHEMICAL DIFFERENCES BETWEEN NORMAL AND
ABNORMAL HEMOGLOBINS (248, 251)

Hemoglobin	Structural characteristics	Chain in which structures occur
A	His·Val·Leu·Leu·Thr·Pro·*Glu*·Glu·Lys	α
S	His·Val·Leu·Leu·Thr·Pro·*Val*·Glu·Lys	α
C	His·Val·Leu·Leu·Thr·Pro·*Lys*·Glu·Lys	α
E	Lys replaces Glu at locus different from S and C	α
D₁	Differs at same locus as E, exact amino acids involved not known	α
D₀⎫	These two are much alike; possibly differ at same locus although not at the same locus as S and	
T ⎭	C or D₁ and E	β

resistant "core," poor in these amino acids but rich in aromatic and neutral
amino acids. The soluble peptides were examined by a "fingerprint" tech-
nique involving electrophoresis on paper in one dimension followed by
chromatography in the other. Comparison of the patterns from digests of
hemoglobin A (normal) and S (sickle-cell)showed that all peptides were
the same, except for one which was in a different position in each case.
These two peptides were shown to differ in a single amino acid residue.
These findings are given in Table VI which includes the known structural
differences in the various types of hemoglobins. Hunt & Ingram (354)
showed by hydrolysis of the "core" with chymotrypsin and subsequent
analysis of the resulting peptides by "finger printing" that the "cores" of
both hemoglobin A and S were the same. It thus appears that the hemo-
globins A and S differ by only one amino acid and that the difference is at
the same locus in the molecule. Because hemoglobins are thought to contain
two half-molecules, each half molecule containing two identical chains, the
absence of glutamic acid should occur in two chains of a half molecule, a
conclusion supported by the observation that hemoglobin S contains two less

carboxyl groups than A, as judged from the electrophoretic mobility (355). Hemoglobins E and D appear to possess sequence changes at a different site in the molecule than that found with hemoglobins C and S. Although no exact sequence is yet available, a glutamyl residue of hemoglobin A is replaced by a lysyl residue in hemoglobin E. The amino acids involved in D are not yet established. Hemoglobins D_0 and I also have a change in sequence at a different locus than that in either hemoglobins S and C or hemoglobins D_1 and E.

Ingram (348) has been able to separate the two types of hemoglobin chains. One of these designated as α (which contains the Val·Leu amino end-group sequence described above) contains the abnormal sequences of hemoglobins S,C,E, and D_1, whereas the other chain, designated as β contained the abnormalities associated with hemoglobins I and D_0.

Aside from establishing chemical differences among these hemoglobins, these studies provide information concerning the genetic control of hemoglobin synthesis. Since hemoglobins S and C are allelic, that is, the mutations have occurred at the same genetic locus, it is noteworthy that each of these hemoglobins differs in a single residue. The most striking feature of these changes, however, is that they appear to involve only single amino acid residues in a molecule of nearly 300 residues.

Ovalbumin.—Although the transformation of ovalbumin to plakalbumin has been studied in detail, Ottesen (356), using crystalline subtilisin, has recently re-examined this conversion and obtained the same plakalbumin as with the crude enzyme. Careful measurement of the reaction at pH 8 showed that the first step of the transformation involved the hydrolysis of a single peptide bond of ovalbumin and that this was followed by hydrolysis of two more bonds, resulting in the liberation of peptide material and the plakalbumin. Because of the small amount of enzyme used, it was possible to confirm definitely that the peptides released had the sequences Ala·Ala and Glu·Ala·Gly·Val·Asp. Accordingly, it was possible to write a schematic picture of the events in the conversion (Figure 2).

It is interesting that the product produced after hydrolysis of the first peptide bond will not crystallize as plakalbumin; however, removal of up
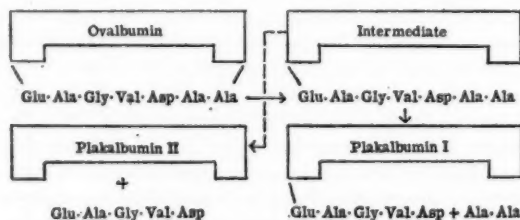
FIG. 2. Ovalbumin-plakalbumin transformation.

to seven residues from the new C-terminal sequence permits crystallization of the modified protein.

Fibrinogen.—Considerable effort has been made in recent years to determine the mechanism of the fibrinogen to fibrin conversion; a comprehensive review of these studies has been given by Scheraga & Laskowski (357). By chemical modification of reactive side chains of fibrinogen, some information has been obtained concerning the role of certain groups in the clotting process. Caspary (358) has acetylated fibrinogen with N,S-diacetylthioethanolamine, which is believed to react only with free amino groups. Acetylation of 35 per cent of the available amino groups resulted in a product which was no longer clottable by thrombin, even though a peptide was split off the acetylated material. In addition, the acetylated fibrinogen appeared to inhibit competitively the thrombin conversion of fibrinogen to fibrin. The author suggests that the acetylation decreases the net positive charge on fibrinogen, thus reducing the ease of polymerization which can be viewed in part as an electrostatic process.

Fitzgerald & Koltun (359) have studied the reaction of *p*-tolyl-diazonium salts with fibrinogen. They believe that less than 20 per cent of the diazonium salt reacts with tyrosine and histidine through the azo linkage, while some salt is reversibly bound and some reacts with other amino acids in an irreversible linkage. Although coupling occurred to the extent of 50 *p*-tolylazo groups per mole of fibrinogen, neither the basic structure nor the clotting process was markedly altered.

Reports have also been made concerning the primary structural changes which occur during the conversion of fibrinogen to fibrin. Gladner, Folk & Laki (360) have reported the isolation and partial characterization of the two peptides released from fibrinogen after the thrombin-catalyzed conversion to fibrin. Both peptides are acidic and have minimum molecular weights of 5400 and 2500, respectively. On the basis of partial sequence determination of these peptides, the authors conclude that thrombin possesses a selective specificity for an arginyl-glycyl bond in the sequence Arg·Arg·Gly.

Blombäck & Yamashina (361) have determined the N-terminal amino acids of fibrinogen and fibrin, as well as the N-terminal residues which appear during the thrombin-catalyzed conversion. Fibrinogen contains two N-terminal glutamic acid and two tyrosine residues per mole. Because fibrin contains two tyrosine and four glycine N-terminal residues per mole, it seems that the four glycines of fibrin replace the two glutamic acid residues of fibrinogen. Also, since glycine appears as a new end group in soluble protein material during conversion, but disappears after complete conversion, the authors conclude that fibrinogen transformation to fibrin includes the production of a soluble fibrin before it polymerizes to the insoluble clot.

Wallen & Bergström (362) have reported the changes which occur in the end groups of fibrinogen after lysis with plasmin previously activated with urokinase. In contrast to the end groups found after thrombin action

on fibrinogen, these workers show the appearance of a total of ten new end groups.

Glycoproteins.—Although it has long been known that the carbohydrate moiety of several glycoproteins is firmly bound to the protein, the nature of such linkages has remained unknown.

The structure of glycopeptides obtained from human γ-globulin has been reported by Rosevear & Smith (363). Peptides were obtained by digestion with papain and subsequent purification by ion-exchange chromatography, alcohol precipitation and zone electrophoresis. Three major glycopeptides were isolated. The largest peptide contained, in round numbers, the following residues: eight hexose, six glucosamine, two fucose, one sialic acid, two aspartic acid, three glutamic acid, and one tyrosine. The other peptides contained the same or lesser amounts of these residues and are believed to be degradation products derived from the same sequence. On the basis of end-group analysis of the three glycopeptides by the fluorodinitrobenzene method, as well as examination of the amino acids released by leucine aminopeptidase, the sequence for the largest peptide is Glu·Glu·Asp (NH_2)·Tyr·Glu·Asp(carbohydrate), the carbohydrate being linked to aspartic acid, probably through the β-carboxyl group, by amide or ester linkage.

Johansen, Marshall & Neuberger (364) are continuing an investigation of the linkage between the carbohydrate and protein moieties of ovalbumin —a study initiated many years ago by Neuberger (365). Heat-denatured ovalbumin was hydrolyzed with pepsin, trypsin, chymotrypsin, and mold protease. Fractionation and purification of the digest by column chromatography and finally by column electrophoresis yielded a purified peptide containing five mannose, three glucosamine, approximately one residue each of leucine and aspartic acid, and about 0.5 residues of serine and threonine. End-group analysis demonstrated N-terminal aspartic acid. A product containing only carbohydrate and aspartic acid was obtained after digestion with carboxypeptidase. The authors conclude that the carbohydrate is linked directly to one of the carboxyl groups of aspartic acid, while the other carboxyl group is linked to leucine. A structure compatible with their work would be: H_2N·Asp(Carbohydrate)·Leu·(Ser,Thr)COOH.

Jevons (366) has reported isolation of a glycopeptide from ovalbumin digests after prolonged hydrolysis with a crude pancreatic extract. The peptide contained mannose, glucosamine, leucine, and aspartic acid in the ratio 4 : 2 : 1 : 1. Only traces of other amino acids were present. Although no suggestion was made concerning the sequence of this material, the results are in agreement with those described above.

Cunningham *et al.* (367) have prepared glycopeptides from the *p*-mercuribenzoate derivative of ovalbumin by digestion with trypsin and chymotrypsin. Fractionation of these digests, followed by cellulose column chromatography yielded a glycopeptide which contained mannose, glucosamine, tyrosine, and leucine in the ratio of 4 : 2 : 1 : 1. Tyrosine was N-terminal

in this peptide and leucine, valine, threonine, serine, and aspartic acid were also present. Another glycopeptide, which was obtained after extensive chymotryptic digestion, contained N-terminal aspartic acid as well as N-terminal tyrosine. A tentative structure of the glycopeptide was presented as: H_2N-Tyr,Asp(Carbohydrate)·(Thr,Ser,Val)Leu-COOH which, for the most part, is consistent with the results of Johansen et al. and Jevons described above.

Muir (368) has examined the nature of the linkage between protein and carbohydrate in a chondroitin sulfate complex. This complex, obtained by extraction of hyaline cartilage, after purification was shown to be electrophoretically homogeneous and to contain about 10 per cent protein. The protein material was particularly rich in dicarboxylic acids and serine. After digestion with papain, the complex contained about half its original amount of serine, whereas the quantities of other amino acids were much less. Although no small glycopeptides were isolated which might indicate the exact nature of the protein-carbohydrate linkage, the author concluded that the complex consists of polysaccharide chains cemented together by polypeptide units. In addition, the linking bond exhibited an alkali sensitivity similar to that of an ester or lactone. Whereas no structural studies have been performed it appears that a similar carbohydrate-protein complex has been isolated by Partridge & Davis (369).

Antibody.—Porter (370) has chromatographically separated three distinct components from a papain digest of rabbit γ-globulin. The molecular weights of these components were about one-third (55,000 to 60,000) that of native γ-globulin (160,000). When the digested γ-globulin contained antibody specific to a purified antigen, e.g., ovalbumin, two of the components (I and II) from this γ-globulin would specifically inhibit the ovalbumin-antiovalbumin combination, but would not inhibit other antigen-antibody reactions. The third fraction (III) would not combine with a homologous antigen, whatever the type of antisera from which it was obtained. On the other hand, fraction III was antigenic and reacted with an anti-γ-globulin serum. In addition, it was readily crystallized, unlike whole γ-globulin which has never been crystallized. Fractions I and II were not active in this respect. Porter suggests that γ-globulin consists of three parts: fraction III is apparently identical in all γ-globulins and is responsible for the common antigenic specificity. Fractions I and II contain the antibody combining centers which presumably vary from one molecule to another.

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PROTEIN BIOSYNTHESIS[1,2]

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It has not been possible to deal with all aspects of the biosynthesis of proteins in this review. The topics discussed largely represent the interests of the reviewer, and no claim is made that the review is comprehensive. The genetic control of protein biosynthesis is discussed by Fincham elsewhere in this volume (343-64). For more general reviews of protein biosynthesis the reader is referred to a previous article in this series by Chantrenne (1) and to a recent review by Loftfield (2). Many articles pertinent to protein biosynthesis appear in two recent symposia: *The Chemical Basis of Heredity* (3) and "The Biological Replication of Macromolecules" (4). Campbell has recently reviewed protein synthesis with special reference to growth processes, both normal and abnormal (5).

STAGES PRIOR TO FORMATION OF POLYPEPTIDE

Source of amino acids.—It now seems very probable that, in general, amino acids, and not peptides, are used for the synthesis of proteins. For example, although free peptides of small molecular weight have been shown to occur in yeast by McManus (6) and by Turba & Esser (7), and in *Pseudomonas hydrophila* by Connell & Watson (8), there is no evidence that these compounds are utilized as such for protein synthesis. Although Šorm & Rychlík (9) reported that enzymic hydrolysates of proteins stimulated increases in enzyme activity in mouse pancreas slices, more recent work has shown that the stimulation probably results from the action of glutamine and asparagine derived from the peptides [Rychlík & Šorm (10)]. The provision of glutamine and asparagine presumably meets a requirement for these compounds as such for protein synthesis, since these amides are incorporated into protein independently of the corresponding amino acids [see, for example, Barry (11); Sansom & Barry (12); Levintow, Eagle & Piez (13)]. Simi-

larly, as discussed in a later section, there is in most cases little evidence for the participation of free peptide precursors of large molecular weight in protein synthesis.

Several recent publications have indicated that amino acids might sometimes be provided for protein synthesis, not from the free amino acid "pool" of the cell, but in some form not in ready equilibrium with this pool. It has been suggested that compounds such as proteins, peptides, or keto acids can give rise directly to forms of amino acids which do not equilibrate extensively with free amino acids present but which are probably "activated" and available for protein synthesis. Support for such a view comes, in part, from the results of Walter & Mahler (14) on the utilization of labelled protein, peptides, or amino acids for protein synthesis in chick embryos, from those of Korner & Tarver (15) on the degradation of the protein of rat liver subcellular fractions *in vitro,* and from those of Shive, Dunn and their co-workers on the utilization of peptides and keto acids in bacteria [see Shive & Skinner (16)].

Alternative explanations of these experiments are, however, possible. Walter & Mahler (14) recognize that their results could also be explained by protein degradation and synthesis occurring at sites located so closely together as to prevent equilibration with free amino acids, although they do not favour this hypothesis. Interpretation of the studies of Shive *et al.* is complicated by factors such as competition for entry into the cell. Halvorson & Cohen (17) have shown that, in yeast, exogenous amino acids appear to be utilized for protein synthesis without equilibrating with the intracellular pool of free amino acids.

Amino acid activation.—The existence of enzymes which catalyse the carboxyl activation of amino acids was first reported by Hoagland (18). As discussed below, enzymes of this type catalyse the reaction between the carboxyl group of an amino acid and ATP, with the resulting formation of an amino acid adenylate and PP. The reaction may be measured by the exchange of ^{32}PP with ATP or by the formation of hydroxamate after reaction of the activated compound with hydroxylamine. Such enzymes are widely distributed, and a number have been purified to varying degrees [see Chantrenne (1); Novelli (19), and individual references below (20 to 31, 35)]. Hoagland (18) originally showed that activating enzymes occur in the soluble (cell sap) fraction of rat liver, the enzymes being precipitated at pH 5 (pH 5 fraction). Other work has shown that the enzymes are largely concentrated in the soluble fraction of other cells, although some activity may be associated with particulate structures [see, for example, Davis & Novelli (20); Jencks & Lipmann (21); Webster (22); Weiss, Acs & Lipmann (23)].

It now seems probable that separate enzymes exist for the activation of individual amino acids or groups of related amino acids. There have been, however, contradictory reports as to whether all amino acids do react with ATP in this manner. Some workers have been unable to show the activation

of an appreciable number of amino acids, and in some cases a given amino acid forms a hydroxamate but does not stimulate PP-ATP exchange to an equivalent extent or does not even stimulate the exchange at all [Novelli (19); Davis & Novelli (20); Webster (22); McCorquodale & Mueller (24); Schweet, Holley & Allen (25); van de Ven, Koningsberger & Overbeek (26)]. On the other hand, some workers have been able to obtain some stimulation of PP-ATP exchange by most or all of the natural amino acids [Novelli (19); Beljanski & Ochoa (27); Bernlohr & Webster (28); Clark (29); Lipmann (30); Nismann, Bergmann & Berg (31)].

The reason for these discrepancies is not yet clear. Some activating enzymes are certainly less stable than others. Nismann *et al.* (31) have shown that, upon storage, the activity of some enzymes decreases more rapidly than that of others. In many systems, particularly crude extracts, there is a high level of endogenous activity, i.e., failure to show activation of a given amino acid often means that activation cannot be measured above a high basal value. Novelli (19) and Novelli & DeMoss (32) suggested that only certain amino acids might be activated by reaction with ATP, other amino acids being activated by secondary anhydride exchange. There is, however, no direct evidence for this.

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larly, as discussed in a later section, there is in most cases little evidence for the participation of free peptide precursors of large molecular weight in protein synthesis.

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but smaller than the soluble RNA derivatives discussed below. On the other hand, Boeyé (41) was unable to find appreciable amounts of activated glycine compounds in intact yeast by a hydroxylamine-trapping technique.

While the tryptophan-activating enzyme shows a high specificity in its substrate requirement for reaction with ATP, it can utilize many synthetic adenylates for the reverse reaction [Novelli (19); Karasek *et al* (37)]. This is clearly an unexpected finding. Novelli (19) has suggested that it might result from contamination of the enzyme with a bound form of tryptophan and the existence of anhydride exchange between the bound amino acid and the amino acid of the adenylate.

A number of the features of activating enzymes of the Hoagland type, such as their apparent failure to catalyse the activation of all amino acids in some systems, have raised the question whether this represents the only method by which amino acids are activated. Certainly, fractions containing these enzymes must be added to obtain appreciable labelling of protein in a variety of systems [see Webster (22); Keller & Zamecnik (42); Littlefield & Keller (43)]. The recent study of Boyer & Stulberg (44) in which ^{18}O-labelled amino acids were used also supports the involvement of this pathway in the synthesis of protein *in vivo* in *Leuconostoc mesenteroides*. On the other hand, Beljanski & Ochoa (27) have obtained a particulate preparation from *Alcaligenes faecalis* in which there is incorporation of amino acids into protein in the apparent absence of activating enzymes for catalysis of the PP-ATP exchange. Some kind of activation takes place, however, as incorporation is dependent upon the occurrence of oxidative phosphorylation. An enzymatic component which promotes the incorporation of amino acids was partially purified. Further work is required to settle this problem and to discover the significance of the findings of Beljanski & Ochoa.

The formation of amino acid adenylates by no means represents the only way in which amino acids could be activated. Some amino acid-dependent exchange of orthophosphate with ATP has been reported to occur in microorganisms [Novelli (19); Eggleston (45)], but the relationship of this exchange to protein synthesis is not clear. The general problem of amino acid activation has been reviewed by Wieland & Pfleiderer (46), with discussion of the detection, synthesis, and properties of activated compounds.

Vitamin B$_{12}$ and protein biosynthesis.—Wagle, Mehta & Johnson (47) reported that in B$_{12}$-deficient rats or pigs, the incorporation of ^{14}C-serine or the carbon of ^{14}C-glucose into liver protein was decreased to about half that found in control animals. There was also a much lower level of incorporation of ^{14}C-methionine, -alanine or -phenylalanine into the protein of microsome-cell sap preparations from the livers or spleens of B$_{12}$-deficient rats. The addition of B$_{12}$, *in vitro*, to these systems largely restored the level of incorporation to control values and also slightly stimulated incorporation in control systems. In two preliminary communications, Wagle, Mehta & Johnson suggested that B$_{12}$ is a cofactor for enzymes concerned in amino acid activation. Thus, when pH 5 fractions from either control or B$_{12}$-de-

ficient rats were added to microsome material from deficient rats, it was found that the system containing the control pH 5 fraction had the higher rate of incorporation (48). After ^{60}Co-vitamin B_{12} had been administered, they found that most of the radioactivity present in the pH 5 fraction was concentrated in a subfraction which contained most of the PP-ATP exchange activity [Wagle, Mehta & Johnson (49)]. The addition of an anti-B_{12} to this subfraction reduced the rate of PP-ATP exchange. They did not state, however, whether this exchange was dependent upon the presence of added amino acids.

Although we have confirmed the observation that amino acids may be incorporated into protein at reduced rates in various systems from B_{12}-deficient rats, we have been unable to obtain any appreciable stimulation of incorporation by the addition of B_{12} in vitro [Arnstein & Simkin (50)]. Fraser & Holdsworth (51) have repeated most of the experiments of Wagle et al. using B_{12}-deficient chicks, but they also have been unable to obtain appreciable stimulation of incorporation in most instances by the addition of B_{12}, or to confirm a number of other findings. It is not yet clear what is responsible for these discrepancies. Differences in the conditions of some experiments, resulting, for example, in differences in incorporation rate, may provide part of the explanation. The question as to whether B_{12} has a direct or an indirect role in protein synthesis remains to be answered by further work. The more general aspects of this problem are dealt with by Coates & Porter elsewhere in this volume. (439-66).

Soluble RNA.—Hoagland, Zamecnik & Stephenson (52) first reported that if the pH 5 fraction of the cell sap of rat liver, which contains amino acid-activating enzymes, was incubated with ATP and a ^{14}C-amino acid, the amino acid became bound to the RNA present. They have symbolized this soluble RNA as SRNA. The amino acid bound to the SRNA could be transferred to microsome protein. They suggested that SRNA serves as an intermediate stage between activated amino acid and ribonucleoprotein. Similar material has since been reported to occur widely: in rabbit liver [Ogata & Nohara (53)], guinea pig liver [Schweet et al. (54)] Ehrlich ascites cells [Hoagland et al. (55), Zamecnik, Stephenson & Hecht (39)], pigeon pancreas [Weiss, Acs & Lipmann (23)], *Tetrahymena pyriformis* [Mager & Lipmann (56)], *Escherichia coli* [Berg & Ofengand (57)], and possibly in *Staphylococcus aureus* [Gale, Shepherd & Folkes (58)] and in pea seedling ribonucleoprotein particles [Webster (22, 59)].

There seems to be a general similarity in the properties of the SRNA from different sources. As noted above, SRNA can be labelled with an amino acid by incubation of a pH 5 fraction with amino acid and ATP. Hoagland et al. (55) found ATP to be specific for this reaction. Alternatively, purified activating enzymes may be substituted for the pH 5 fraction and isolated RNA used as acceptor [Berg & Ofengand (57); Schweet et al. (54)]. There is a requirement for some specificity in the RNA used as acceptor. This specificity is not, however, absolute, for Schweet et al. (54)

have found that the RNA acceptor does not seem to be species specific [cf. Mager & Lipmann (56)]. It has been suggested that the amino acid moiety of the amino acid adenylate formed by the activating enzyme is transferred to SRNA:

$$\text{amino acid} + \text{ATP} \rightleftharpoons \text{amino acid—AMP} + \text{PP} \qquad 1.$$
$$\text{amino acid–AMP} + \text{SRNA} \rightleftharpoons \text{amino acid–SRNA} + \text{AMP} \qquad 2.$$
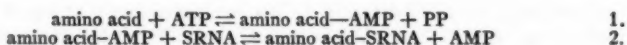
Berg & Ofengand (57) suggested that the formation of the adenyl anhydride and the subsequent transfer of the amino acid moiety to the acceptor were carried out by a single enzyme, since on purification of the system, PP-ATP exchange activity for valine or methionine increased to the same extent as the ability to incorporate amino acid into SRNA.

Studies on the reversibility of the labelling of SRNA support the hypothesis that these two reactions are somehow linked. Thus, Mager & Lipmann (56) found that loss of amino acid was enhanced by the addition of both AMP and PP. They suggested that the inability of AMP alone to cause appreciable reversal could indicate that amino acid adenylate is not a free intermediate (as suggested by other studies, see above) or that reversal of reaction 2 is energetically not feasible without the simultaneous reversal of 1. Berg & Ofengand (57) also found that amino acids were readily removed from SRNA in the presence of AMP and PP, and Hoagland et al. (55) found a loss of label in the absence of ATP which was increased in the presence of PP.

Before the occurrence of SRNA had been reported, Holley (60) had found that there was an incorporation of labelled AMP into ATP in the presence of the pH 5 fraction of rat liver which was dependent on the presence of alanine. Other amino acids did not appear to stimulate the exchange. This exchange may be associated with SRNA; it is sensitive to ribonuclease, as is the labelling of SRNA. Webster (22) found a similar AMP-ATP exchange to be associated with ribonucleoprotein particles from pea seedlings, but in this case amino acids other than alanine stimulated the exchange.

Each amino acid appears to be linked to SRNA independently of others and there seems to be a limiting level for each amino acid [Schweet et al. (54); Hoagland et al. (55); Berg & Ofengand (57)]. This suggests specific binding sites for each amino acid. Sites for different amino acids might exist on the same SRNA molecule, or there may be different SRNA acceptors for different amino acids. Schweet et al. (54) reported preliminary evidence for an SRNA fraction which could accept leucine but not tyrosine. The various studies cited above (23, 54, 55, 57) have revealed some of the properties of the amino acid-SRNA compounds. Amino acids are linked to SRNA by covalent bonds, and possible modes of linkage have been discussed by Berg & Ofengand (57) and by Hoagland et al. (55). It appears that SRNA must have a certain terminal nucleotide sequence before it can act as an amino acid acceptor. Hecht, Stephenson & Zamec-

nik (61) have found that unless the SRNA of ascites cells has a terminal adenine nucleotide and cytosine nucleotides adjacent to this end group, there is little capacity to incorporate any of 14 amino acids tested. This similarity of requirement is somewhat surprising in view of the apparent specificity of the binding of different amino acids and presumably indicates that specificity resides elsewhere in the molecule.

Hoagland et al. (55) have made a study of the transfer of amino acid from SRNA to microsome protein. The transfer of ^{14}C-leucine from the pH 5 fraction of rat liver required a nucleoside triphosphate-generating system and GTP specifically [cf. Keller & Zamecnik (42)]. ATP failed to stimulate the transfer, and the addition of ^{12}C-leucine failed to affect the transfer of label. When isolated leucine-SRNA was used, a partial requirement for ATP could be shown. A requirement for an enzymatic component of the pH 5 fraction was also shown [see also Zamecnik, Stephenson & Hecht (39)]. Webster (22) has also reported some stimulation of the incorporation of amino acid into the protein of pea seedling or yeast ribonucleoprotein particles by GTP.

Zamecnik's group has examined the criteria for the hypothesis that SRNA is an obligatory intermediate in protein synthesis, and the available evidence is consistent with this hypothesis [Zamecnik, Stephenson & Hecht (39); Hoagland et al. (55)]. They have, however, pointed out that the possibility that SRNA might have a function as a storage site of activated amino acids cannot be completely excluded.

The amino acids attached to SRNA do not appear to be present in peptide linkage [see, for example, Hoagland et al. (55)]. However, Koningsberger, van der Grinten & Overbeek (62) have reported the occurrence in bakers' yeast of dialysable compounds which appear to be peptides bound in some way by carboxyl linkage to nucleotides. Dirheimer, Weil & Ebel (63) also have briefly described the occurrence of similar compounds in bacteria, mushrooms, and rabbit tissues. While such compounds may represent intermediates in the protein synthetic pathway, perhaps serving in the transport of activated amino acids to ribonucleoprotein [Koningsberger et al. (62)], there is as yet no direct evidence that they are in fact involved in protein synthesis.

POLYPEPTIDE FORMATION

There are three main ways in which amino acid residues could be linked together to form the polypeptide chain of a protein: (a) amino acid residues could be joined together to form peptides which are later linked together with other peptides or amino acids to form the complete protein chain; (b) all of the amino acid residues might be linked together simultaneously after adsorption on a preformed catalytic site, or (c) the amino acids could be linked together in a definite sequential manner, beginning at one end of the polypeptide chain and proceeding to the other. The manner in which the sequence of amino acids is determined, the way in which polymeriza-

tion is catalysed and the intracellular location of polypeptide synthesis are considered in other sections of this review.

Most of the available evidence does not favour the existence of long-lived peptide intermediates in other than trace quantities [see recent reviews by Spiegelman (64) and Cohn (65)]. Several recent papers, such as those of Stavitsky (66), Stavitsky & Wolf (67), and Taliaferro & Taliaferro (68) on antibody synthesis, and of Pollock & Kramer (69) on penicillinase synthesis in *Bacillus cereus* provide further support for this view. There may, however, be exceptions to this generalization. Raacke (70) suggested that peptide intermediates are involved in the synthesis of pea seed proteins, but the possibility that the peptides are broken down prior to utilization cannot be entirely excluded.

It has been suggested that a precursor, presumably of high molecular weight, is involved in the synthesis of the extracellular amylase of *Bacillus subtilis* [Nomura *et al.* (71, 72)]. Straub and his colleagues also have proposed that a precursor of this type is involved in the synthesis of pancreatic amylase. Ullmann & Straub (73, 74, 75) found that increases in amylase activity could be obtained in various cell-free systems prepared from pancreas, but later work with isotopic amino acids indicated that this phenomenon did not represent the synthesis of amylase, *de novo*, from amino acids [Garzó *et al.* (76)]. The addition of threonine, arginine, and a high concentration of ATP is required to obtain increases in amylase activity in cell-free systems from pigeon pancreas [Ullmann & Straub (77)]. Some other information concerning these systems has been given by Straub (78). From the results of experiments with isotopically labelled amino acids, Straub has proposed that amylase precursor is synthesized in the microsome material and that its conversion to amylase takes place in "large granules" [Garzó, T-Szabó & Straub (79); T-Szabó & Garzó (80); Ullmann, Garzó & Straub (81)]. This is not the only conceivable interpretation of these findings, and the possibility that the phenomenon represents the release of amylase from some bound form cannot be excluded. The amylase-distribution studies of Laird & Barton (82, 83) support the hypothesis that amylase is synthesized completely by the microsome material, but their evidence is less direct than that of Straub and his colleagues.

Hendler (84) has also reported the occurrence of some intermediate stage in protein synthesis between free amino acid and TCA-insoluble protein in hen oviduct. The nature of the intermediate compounds is not clear: possibly amino acids bound to SRNA (see above) or to lipide (see below) are involved.

Some years ago, several groups investigated whether, following exposure to an isotopically labelled amino acid, a given amino acid residue had the same specific activity at different loci along a polypeptide chain. Such data might provide information concerning the occurrence of peptide intermediates. While studies in this and other laboratories failed to find evidence for unequal labelling, Anfinsen and his colleagues did present

evidence for the unequal labelling of several proteins [see review by Steinberg, Vaughan & Anfinsen (85)]. The causes of this discrepancy are not obvious. Recently, Kruh *et al.* (86) have found unequal labelling of the glycine residues of rabbit haemoglobin, labelled *in vitro* or *in vivo*. Earlier Muir, Neuberger & Perrone (87), in experiments with ^{14}C-valine had not detected unequal labelling of rat haemoglobin *in vivo*. The interpretation of experiments of this type is difficult, and Kruh *et al.* have considered some possible explanations of their work. Further experiments are required before a definitive interpretation can be made.

If the assumption is made that long-lived free intermediates are not involved in the process of polymerization, then in order to obtain information about the mechanism of protein synthesis it becomes essential to study the labelling of proteins at very short intervals after exposure to a labelled amino acid. It has been found in many investigations that there is little or no delay in the incorporation of isotope into nonspecific cell protein (i. e., total TCA-precipitable or saline-insoluble protein). However, several exceptions to this generalization have been reported. For example, there is little delay in the appearance of label in amino acids in yeast, on incubation with ^{14}C-acetate, but a considerable delay in appearance in protein [McManus (6); Turba, Leismann & Kleinhenz (88)]. The interpretation of these findings is complicated by the recent work of Halvorson & Cohen (17) mentioned earlier. In *A. vinelandii* there is also a delay, although brief, before appearance of isotope in protein [Bernlohr & Webster (40); Burma & Burris (89)]. On the other hand, in the case of soluble proteins isolated after secretion from the cell, there are numerous reports of a delay in the appearance of label. Examples of proteins for which this has been found include: serum albumin [see Green & Anker (90); Peters (91, 92)]; antibody [Taliaferro & Taliaferro (68); Askonas & Humphrey (93, 94); Humphrey & Sulitzeanu (95)]; Bence Jones protein [see Putnam (96)]; myeloma protein [Nathans, Fahey & Potter (97)], oviduct proteins [Hendler (98)], and pancreatic juice proteins [Rothschild, Hirsch & Junqueira (99)].

Recent work has indicated that this delay in the labelling of specific secreted proteins is the result of two or possibly three factors: (*a*) Delay in the secretion of labelled protein from the cell [Peters (92); Askonas & Humphrey (93, 94); Humphrey & Sulitzeanu (95); Nathans, Fahey & Potter (97)]. (*b*) Delay in the release of labelled protein from the site of synthesis. This is most clearly shown by the work of Peters (92) on albumin formation in liver. He found that a protein with properties identical with those of albumin could be released from cytoplasmic particles by treatment with deoxycholate. This protein was labelled before label appeared in the albumin of the soluble phase of the cell. (*c*) The time required to synthesize the specific protein molecule. The available evidence relating to this is somewhat contradictory. The investigations of Pollock & Kramer (69) on the exopenicillinase of *B. cereus* and of Craddock & Dalgliesh (100) on the

ribonuclease of rat pancreas suggest that if there were any delay in the attainment of the maximum labelling of these proteins, it would have to be of very short duration—perhaps less than 30 sec. Peters (92) estimated that the labelling of the bound albumin of liver particles did not require more than 2 to 3 min. Garzó, T-Szabó & Straub (79) reported a delay of 5 to 10 min. in the labelling of what they believed to be the total cellular amylase of pigeon pancreas slices. Amylase may not, however, be an ideal protein for this type of study in view of the possible involvement of a complex precursor in its synthesis (see above).

Loftfield & Eigner (101) found that while peptide-bound leucine or valine was present in rat liver ferritin as early as 20 sec. after injection of amino acid, at least 4 to 5 min. were required for maximal labelling to be obtained. They proposed that this time represents that required for the over-all synthesis of a single ferritin molecule, including peptide-bond formation and secondary processes such as cross-linking and folding. The possibility should be considered that some part of this estimated time of synthesis is required for dissociation from a bound form. Further information on the time required for the synthesis of protein molecules might help to distinguish between mechanisms involving simultaneous formation of all peptide bonds and those involving sequential addition. Studies on unequal labelling at very short time intervals might also help to distinguish between possible mechanisms.

Several observations suggest that the release of bound protein is a metabolic process. Peters (92) found that both the release of albumin from cytoplasmic particles and secretion from the liver cell were inhibited by cyanide or dinitrophenol. Rabinovitz & Olson (102, 103) have briefly reported that soluble proteins are released from reticulocyte ribonucleoprotein in a cell-free system and that the process is stimulated by ATP. In a study of the labelling of cell sap protein in a microsome-cell sap system from liver, the reviewer has shown that there is, after an initial delay, transfer of label from microsome to cell sap protein [Simkin (104)]. The transfer appears to be associated with an ATP-requiring process. Hendler (105) showed a transfer of radioactivity from a ribonucleoprotein-containing fraction (from oviduct debris) to soluble protein. The appearance of labelled soluble protein was stimulated by the presence of ATP, GTP, and CoA. It is not clear, however, whether these cofactors are required for the conversion of bound amino acids present in the debris to protein [Hendler (84)] or for the release of formed protein.

Association of lipides with protein biosynthesis.—Hendler (106) has suggested that lipides might play some part in protein synthesis in hen oviduct. Lecithinase A, lysolecithin, and deoxycholate inhibited the incorporation of amino acids into the protein of oviduct minces, whereas cytosine triphosphate and CoA stimulated incorporation. Perhaps it is essential that the structural integrity of the endoplasmic reticulum, which has a high lipide content, be maintained for optimal protein synthesis in this

system. Zamecnik & Keller (107) had earlier failed to find an appreciable effect of CoA, although at lower concentrations, upon amino acid incorporation into protein in a cell-free system from rat liver. Hendler also found that the chloroform-soluble lipide fraction of oviduct contained a significant amount of radioactive amino acid: this was apparently not in a free form and was liberated upon hydrolysis. Amino acid appeared to enter and leave this lipide-bound fraction rapidly. Further work is clearly required to establish whether lipides do have a direct role in protein synthesis.

Sites of protein biosynthesis.—Earlier work on this subject has been adequately discussed in a number of reviews [for example, Chantrenne (1); Loftfield (2)]. Further evidence which demonstrates that the ribonucleoprotein-containing microsome fraction is an important site of cytoplasmic protein synthesis has been provided, by studies, among others, on homogenates of young pigeon pancreas [Weiss, Acs & Lipmann (23)] or of the protozoon *Tetrahymena pyriformis* [Mager & Lipmann (56)]. Other findings reinforce the suggestion that the microsome material is the site of synthesis of proteins which are destined for secretion by the cell. For example, Peters (92) has found that the microsome fraction appears to be the main site of synthesis of albumin in liver. From a study of the intracellular distribution of proteolytic enzyme and ribonuclease activity in guinea pig pancreas, Siekevitz & Palade (108) have suggested that the microsome fraction could be the site of synthesis of these enzymes, but they recognize the dangers of artifacts inherent in such investigations. Laird & Barton (82, 83) made a similar suggestion following a study of amylase distribution in mouse pancreas. As already discussed, Straub and his colleagues believe that amylase is not synthesized completely by the microsome material, but their evidence is not conclusive.

It does seem questionable, however, that those components of the cytoplasm which give rise to the microsome fraction synthesize all of the protein produced in the cytoplasm, although they are clearly responsible for a large part of this synthesis in some cells. Protein synthesis can probably occur in other cytoplasmic structures and, in fact, Bates, Craddock & Simpson (109) have recently shown that mitochondria can synthesize protein, including a specific protein, cytochrome-*c*. There are exceptions to the generalization that the microsome fraction shows the highest rate of protein synthesis. Several of these exceptions were noted by Chantrenne (1) [see also Walter & Mahler (14)]. They are no doubt, at least in part, reflections of the capacity of nonmicrosomal structures, including the nucleus, to synthesize protein.

There is little information concerning the location of protein synthesis in bacterial cells. The recent work of Aronson & Spiegelman (110) and of Gale, Shepherd & Folkes (58) provides some indication that ribonucleoprotein-containing cellular components are involved. Butler, Crathorn & Hunter (111) have proposed that protein synthesis in *Bacillus megatherium* occurs at sites associated with the cell membrane (i.e., protoplast mem-

brane). Incorporation of amino acids occurred even in isolated membrane fractions, and there was a surprisingly rapid transfer of label from membrane material, labelled *in vitro*, to added "cytoplasmic protein." Little transfer occurred, however, when the membrane fraction was labelled in the presence of the cytoplasmic protein. It should be pointed out that Butler *et al.* measured only the incorporation of amino acids into material insoluble in hot TCA. Recent evidence (mentioned below) indicates that this may not always be a sufficient criterion of protein synthesis in bacteria. Further work on this problem is required, particularly as these workers have briefly reported the occurrence of some incorporation of alanine into the cell-membrane fraction in the presence of chloramphenicol [Crathorn & Hunter (112)].

Protein biosynthesis in cell-free systems.—It is well established that isotopically labelled amino acids are incorporated into TCA-insoluble protein in cell-free systems. Increases in total protein, usually very small, have been claimed to occur in various cell-free systems [e.g., Beljanski & Ochoa (27); Khesin, *et al.* (113); Spiegelman (64); Webster (114)]. The problem remains, however, as to whether the incorporation of amino acids into proteinaceous material does form at least part of the normal process of protein synthesis and whether any specific protein can be synthesized *de novo* from amino acids after destruction of cellular integrity. The reviewer has found marked differences in the labelling of the protein of liver microsome material, depending upon whether the material was labelled in the intact animal or in a cell-free system [Simkin & Work (115)]. The significance of these findings is not clear, but it is possible that labelling in this cell-free system is much slower than in the intact cell, so that the pattern of labelling would represent that in the intact cell at very short intervals after exposure to isotope.

Several recent publications have suggested that there is true synthesis of specific proteins in cell-free systems. In animal systems, and perhaps in other systems, studies are complicated by the possibility that the protein formed is initially bound to the site of synthesis (see above) and may be slowly released, or not released at all, under the conditions that obtain. Thus, Campbell, Greengard & Kernot (116) briefly reported that a microsome-bound albuminlike protein is labelled during incubation of a microsome-cell sap system from rat liver. Albumin present in the cell sap did not appear to be labelled, and the reviewer has failed to find appreciable label in the albumin of the cell sap of a similar system from guinea pig liver (unpublished findings). The inability of Askonas & Humphrey (93) and Stavitsky (117) to detect synthesis of antibody following cellular damage might have resulted from the firm attachment of newly synthesized material to subcellular structures. Bates, Craddock & Simpson (109) found that ^{14}C-amino acids were incorporated into the cytochrome-*c* of isolated mitochondria from rat liver.

There have also been suggestions of the synthesis of specific proteins in

cell-free systems from bacteria. For example, Spiegelman (64) reported increases in the activity of β-galactosidase in disrupted protoplast preparations from *B. megatherium*. A T2 bacteriophage protein antigen was detected by Brown & Brown (118) after incubation of a subcellular system from *E. coli* with bacteriophage DNA. When increase in the biological activity of a protein is used as a criterion of synthesis, the possibility must always be considered that the results obtained are attributable to factors such as destruction of an inhibitor or release of protein from bound form. The increases in amylase activity in preparations of lysed *B. subtilis* described by Nomura, Hosoda & Nishimura (119) probably do not represent *de novo* synthesis, as there is evidence (see above) that a complex precursor is involved in the synthesis of amylase in this organism.

NUCLEIC ACIDS AND PROTEIN BIOSYNTHESIS

It has become obvious that a knowledge of the role played by the nucleic acids in protein synthesis is essential before any understanding can be obtained of the mechanism of protein synthesis and of control of the latter by genetic factors. Any impartial consideration of the available evidence will show that we are still some considerable way from arriving at such an understanding. Only certain recent contributions to this topic will be considered here. An excellent general review of earlier work is to be found in the review by Chantrenne (1) [see also Loftfield (2) and Spiegelman (64)].

Deoxyribonucleic acids.—The genetic control of protein synthesis is ultimately mediated by DNA [see Chantrenne (1); Fincham (pp. 343-64 this volume)]. Nevertheless, as discussed by Chantrenne, interference with DNA synthesis does not, in general, affect the synthesis of protein (or of RNA) [for further demonstrations of this see recent papers by Barner & Cohen (120); Harold & Ziporin (121); Newton (122); Okazaki & Okazaki (123)]. Increase of DNA in *E. coli* B to an abnormally high level did not result in a proportionate increase in the rate of synthesis of proteins, including several enzymes [Pardee & Prestidge (124)]. There was, however, no definite proof that the excess DNA, produced by means of an amino acid analogue, was functional. There is good evidence that protein synthesis can occur even in the absence of DNA [see Chantrenne (1)]. The recent work of McFall, Pardee & Stent (125) does suggest that DNA can have some influence on cytoplasmic protein synthesis. They found that when the DNA of *E. coli* was damaged by ^{32}P radiation, the capacity for the synthesis of both constitutive and induced enzymes was destroyed. The capacity for the synthesis of polypeptide per se and of RNA appeared to be destroyed more slowly.

From the results of an autoradiographic study of the seminal vesicles of mice killed after the administration of ^{14}C-adenine, Pelc (126) has suggested that DNA is synthesized in excess of the need for cell division and that this excess metabolism may be connected with the synthesis of certain proteins. A confirmation of the experimental findings with the use of more

critical methods for the separation of cellular components is required before any assessment of this suggestion can be made.

The recent work of Brown & Brown (118) suggests that it may be possible to study the influence of DNA upon protein synthesis in a cell-free system. A T2 bacteriophage protein antigen was detected in disrupted protoplast preparations from *E. coli* in the presence of T2 DNA. However, the DNA sample was contaminated with 0.3 per cent of protein (apparently not bacteriophage antigen) and, when this was removed, no antigen could subsequently be detected in the cell-free system.

The mechanism by which the genetic information of DNA is transmitted is discussed by Fincham elsewhere in this volume (p. 343ff). Many workers have suggested that DNA could exert its control on protein synthesis by passing information on to RNA which would then control protein synthesis directly. The experimental evidence for this hypothesis is largely indirect, and much more work is required before the hypothesis can be accepted as established. Some recent experiments have added further to the indirect evidence available. Stich & Plaut (127) found that treatment of both enucleate and nucleate fragments of *Acetabularia* with ribonuclease inhibited the capacity for the net synthesis of protein, growth, and differentiation. On removal of ribonuclease, these processes resumed in nucleate fragments but not in enucleate ones. If the influence of the ribonuclease is in fact upon RNA, the results suggest that the capacity to produce RNA, which can then initiate cytoplasmic protein synthesis, resides in the nucleus. Errera & Vanderhaeghe (128) found that after ultraviolet irradiation, enucleate fragments of *Acetabularia* had a decreased capacity for revival and regeneration, compared to nucleate fragments, and they suggested that RNA may play some part in this phenomenon.

Jeener (129) has suggested that when bacteriophage production is induced in a lysogenic strain of *B. megatherium,* DNA transmits information controlling protein synthesis via the formation of a specific RNA. This would explain the inhibition of cell lysis that occurs if ribonuclease, thiouracil, or azaguanine is added before bacteriophage protein appears. When *E. coli* is infected with T2 bacteriophage there is almost complete inhibition of RNA synthesis, but there is, under these conditions, turnover of what appears to be a specific small fraction of the RNA [Volkin & Astrachan (130); Watanabe, Kiho & Miura (131)]. The significance of this phenomenon is not clear. Some protein must be synthesized before T2 bacteriophage DNA can be formed [Burton (132); Tomizawa & Sunakawa (133); Hershey & Melechen (134)]. It might be that the specific RNA fraction is involved in the synthesis of this protein rather than in the production of bacteriophage protein [Watanabe *et al.* (131)].

Ribonucleic acids.—There is now evidence that RNA participates in at least two distinct ways in protein synthesis: (*a*) SRNA is involved at some stage in the transport of activated amino acid residues to the site of protein synthesis; (*b*) ribonucleoprotein RNA is involved at the site of synthesis. Work on protein synthesis by microsomal material has strongly sug-

gested that RNA must be present at the site of synthesis. Since it has been shown, particularly by studies on the RNA of viruses [see, for example, Schramm (135); Fincham (pp. 343-64, this volume)], that specific RNA can influence the specificity of protein synthesis, it is generally assumed that the RNA at the site of synthesis plays a part in the polymerization process, in particular determining the sequence of amino acid residues in the polypeptide chain formed. It should be remembered, however, that the evidence for these functions has been obtained from work on microsomal material; it has not yet been proved that the same mechanism of protein synthesis is operative either in nuclei, where much remains to be discovered about the mechanism of protein synthesis [see Chantrenne (1)], or in other cytoplasmic structures such as mitochondria.

Although there are some clues as to the functions of RNA in protein synthesis, there is virtually no direct information as to the mechanisms involved. Experimental work concerning SRNA is discussed earlier in this review. It is possible that information relating to the exact role of SRNA in protein synthesis will be obtained in the near future. On the other hand, there seems less reason to suppose that direct information concerning the function of ribonucleoprotein RNA will soon be forthcoming. A number of indirect experimental approaches have been tried, but the results of such work are not easy to interpret. The difficulties of interpretation are increased by the failure in many instances to distinguish between different kinds of functionally distinct RNA, only total cellular RNA being investigated. Many hypotheses have been proposed from the results of these indirect investigations, some of them being mutually exclusive. Overinterpretation of data has helped to produce this confusion. There is clearly a need for a much more critical approach to this problem.

One of the most commonly adopted indirect approaches involves measurement of either the net synthesis or the turnover of RNA in relation to protein synthesis. Many of the earlier investigations have been critically discussed by Chantrenne (1) [see also Spiegelman (64)]. The evidence seems to indicate that, in general, the synthesis of RNA must accompany the synthesis of protein in microorganisms. This is particularly apparent in the case of induced enzyme synthesis. Several recent investigations could be interpreted as supporting this view. For example, Ben-Ishai (136) and Borek & Ryan (137) studied protein synthesis in various strains of *E. coli* following upon accumulation of RNA in the absence of protein synthesis. Ben-Ishai found that the presence of the excess RNA did not affect the rate of subsequent protein synthesis, and Borek & Ryan showed that no new protein was synthesized until, after a period of delay, RNA synthesis began again. It may be argued from findings such as these that the mere presence of RNA is not sufficient to promote protein synthesis but that synthesis of RNA is actually required. Before such a hypothesis can be accepted, it must be proved that the apparently functionless RNA at some stage, is actually capable of mediating in protein synthesis.

Okazaki & Okazaki (123) found that a deficiency of uracil in *Lactobacil-*

lus acidophilus caused an inhibition of synthesis of both protein and RNA, but interpretation of this and analogous findings is complicated by the fact that uracil might be required for processes other than the synthesis of RNA. As in a number of other systems [see, for example, Chantrenne (1)], Webster (22) found that a mixture of nucleoside-5′-di- or-triphosphates caused some stimulation of the incorporation of amino acids into the protein of isolated ribonucleoprotein particles. Again, this kind of evidence can be interpreted in several ways—e.g., by the common intermediate hypothesis (see below). There is some evidence which suggests that in fact the synthesis of RNA need not accompany protein synthesis in bacteria. Several recent papers have added to this evidence. For example, Barner & Cohen (120) obtained synthesis of protein in a mutant of *E. coli* in the absence of any detectable net synthesis of RNA (turnover of RNA was not, however, measured); appreciable DNA synthesis did occur during this time. In a preliminary note, Aronson & Spiegelman (110) have reported that if bacteria are treated with chloramphenicol to enable RNA synthesis to occur without concurrent synthesis of protein, subsequent removal of the chloramphenicol permits amino acids to form protein in the absence of detectable RNA synthesis. In any final analysis of this problem, account should be taken of the fact that in many of the bacterial systems used, new sites for protein synthesis are formed during the period of study. It would be desirable to know whether the synthesis of RNA plays a part in protein synthesis over and above meeting this requirement.

This problem has also been investigated in mammalian systems. In these systems studies are not complicated by requirements for cell division and some attempt can be made to separate functionally distinct RNA. It has been found that the RNA of different subcellular fractions has quite different rates of turnover. For example, in liver the RNA of the cell sap, of which SRNA forms a part, has a higher rate of turnover than microsomal RNA [e.g., Shigeura & Chargaff (138, 139)]. Shigeura & Chargaff found that the RNA of ribonucleoprotein particles isolated from the microsome fraction had a lower rate of turnover than other RNA present. These particles are believed to be the site of synthesis of protein in the microsome fraction [Littlefield *et al.* (140)]. Somewhat similar results were obtained in this laboratory with a different method of fractionation [Bhargava, Simkin & Work (141)]. Clark, Naismith & Munro (142) suggested that in rat liver the metabolism of RNA is influenced by the dietary supply of amino acids available for protein synthesis, breakdown of RNA being reduced or abolished when dietary amino acids are being absorbed. Munro & Mukerji (143) have now found that only three amino acids, leucine, glycine, and methionine, are responsible for this phenomenon, but it seems that administration of these amino acids does stimulate the synthesis of protein in the liver, perhaps by some hormonal mechanism. Clearly, in mammalian as in other systems, further work is required, e.g. on the functional significance of RNA present in different parts of the cell.

Another method of studying the involvement of RNA in protein synthesis is to introduce an unnatural purine or pyrimidine base into the cell. The effects of aberrations of nucleic acid and nucleotide metabolism may then be investigated. 8-Azaguanine has been used for this purpose in several recent studies on *B. cereus*. Chantrenne & Devreux (144) found that protein synthesis, as measured by net increase or by incorporation of a number of amino acids into protein, was strongly inhibited by 8-azaguanine, but a number of other cellular syntheses were unaffected, except at high concentrations of the base. Mandel (145) also found inhibition of net synthesis of protein and of the incorporation of ^{35}S-amino acids by 8-azaguanine, but he found a slight increase in the incorporation of ^{14}C from acetate and glutamate into TCA-insoluble material. This might indicate that the incorporation of only certain amino acids is affected, or, alternatively, that label from acetate or glutamate is, in part, incorporated into cell-wall material, the formation of which may be mistaken for protein synthesis unless special precautions are taken (see below). The inhibition of protein synthesis by 8-azaguanine might be the result of the formation of an abnormal RNA. On the other hand, inhibition might result from the presence of an analogue-containing nucleotide or nucleotide derivative. Mandel & Markham (146) found that 8-azaguanosine-5′-phosphate was present in acid-soluble form in *B. cereus* exposed to 8-azaguanine. As mentioned earlier, GDP or GTP plays some as yet unknown role in the transfer of amino acids from SRNA to ribonucleoprotein. An 8-azaguanosine-5′-phosphate might exert an inhibitory effect on protein synthesis at this point.

As a result of the frequent suggestion that the synthesis of RNA is linked to that of protein, much attention has been paid to the mechanism of synthesis of RNA. Such information might give valuable clues as to the mechanism of protein synthesis. A number of workers have found that a full range of amino acids in trace amounts is required for the synthesis of RNA in microorganisms [Gros & Gros (147); Pardee & Prestidge (148); Yčas & Brawerman (149); cf. Barner & Cohen (120); Okazaki & Okazaki (123)]. The function of the amino acids in RNA synthesis is still uncertain. It has been maintained that amino acids are required per se and not as precursors for the synthesis of protein, since RNA synthesis can occur in the presence of chloramphenicol, which inhibits protein synthesis. Aronson & Spiegelman (110) have questioned this view, however; they point out that up to 5 per cent of normal protein synthesis does occur in the presence of the concentrations of chloramphenicol customarily employed. When much higher concentrations of chloramphenicol are used, protein synthesis can no longer be detected. Under these conditions, RNA synthesis was observed in the absence of added amino acids and the addition of amino acids did not result in a marked stimulation of synthesis. Further work is required to settle this problem. One factor that should be taken into account is that the organisms may in fact receive a supply of amino acids from the breakdown of protein (see below).

As stated earlier, there is little direct evidence as to the manner of participation of RNA in protein synthesis. This has not discouraged many workers from exercising considerable ingenuity in devising hypotheses concerning the roles of RNA in protein synthesis. Such speculation has considerable value if it leads to new forms of experimental approach. Many of the speculations put forward in a recent article by Crick (150) seem to be valuable contributions of this kind. For example, it was proposed that SRNA might provide the activated amino acid with an "adaptor" molecule, the adaptor then playing some part in controlling the way in which the amino acids are polymerized together in the correct sequence. It was considered that the adaptor would probably be of nucleotide nature. Hoagland *et al.* (55) have suggested that the whole of the SRNA molecule might serve as such an adaptor.

Nucleotide–amino acid compounds feature in the hypotheses of a number of other workers. As discussed by Chantrenne (1), there are numerous suggestions that nucleotide–amino acid compounds could serve as direct precursors of protein or of RNA, perhaps even serving as common intermediates which would polymerize into either protein or RNA or both, according to the conditions. Much of the available indirect experimental data could certainly be interpreted on the basis of this kind of hypothesis. Michelson (151) has suggested on purely theoretical grounds that compounds formed by the reaction of amino acid adenylates with nucleoside 2′,3′-phosphates would be capable of acting as common intermediates of this type.

There is certainly no lack of hypotheses concerning the way in which amino acids or their derivatives are polymerized together to form a polypeptide chain. Usually the existence of a "template" is postulated, and much consideration has been given to the way in which the sequence of nucleotides in RNA could determine the sequence of amino acids in the protein formed. This subject has been extensively reviewed by several workers [see Chantrenne (1); Fincham (pp. 343-64, this volume)]. Other recent discussions of the problem include those of Crick (150) and Novelli & DeMoss (32). Crick has proposed that what he calls "the central dogma" is a key feature of protein synthesis. This states that once information has passed into protein it cannot be transferred back to nucleic acid. While this may be a useful working hypothesis, much more experimental evidence is required before it can be accepted as a general rule. Koshland (152) has pointed out that the individual features of protein synthesis are those of enzymatically catalyzed reactions and from this he has proposed an ingenious template hypothesis based on the "induced fit" theory of enzyme specificity.

TURNOVER AND EXCHANGE

The concept of the dynamic turnover of proteins was questioned by Hogness, Cohn & Monod (153) who, together with Rotman & Spiegelman (154), showed that the synthesis of β-galactosidase and other proteins in

growing *E. coli* was virtually irreversible. Other work did, however, suggest the existence of some turnover of protein in bacteria and in animal tissues, and recent investigations have demonstrated that turnover can in fact occur.

It has been shown by a variety of independent techniques that there is turnover of protein in nongrowing *E. coli* or yeast [Borek, Ponticorvo & Rittenberg (155); Halvorson (156); Mandelstam (157, 158)]. However, little degradation of protein takes place in growing cells of these organisms, except perhaps at the beginning of the growth period [Halvorson (159); Koch & Levy (160); Mandelstam (158)]. Mandelstam (158) has pointed out that bacterial proteins may differ in lability, β-galactosidase, for example, being relatively stable under conditions where other proteins are being rapidly degraded. In animal systems some definite evidence for protein turnover was obtained in studies on Ehrlich ascites cells [Forssberg & Révész (161); Moldave (162, 163)]. Forssberg & Révész point out that the loss of label from protein varies with the strain of cell and with the amino acid used. The latter finding might be a reflection of different rates of reutilization of liberated amino acids. Moldave (163) found that label was lost from the protein of all subcellular fractions examined. Turnover was more conclusively demonstrated by Harris & Watts (164) in nongrowing rabbit macrophages under *in vitro* conditions. Eagle, Piez & Fleischman (165) found that ^{14}C-phenylalanine or -tyrosine were incorporated into the proteins of various growing tissue culture cells in excess of that accounted for by growth. When net synthesis of protein was stopped by omission of an essential amino acid, some incorporation continued which was reduced by omission of glutamine, but not glucose, and which corresponded to the excess over growth. More recent work supports the view that intracellular turnover of protein is occurring in these cells [Eagle (166)]. A number of other recent studies have also been interpreted as confirming the existence of protein turnover in mammalian cells [e.g. see Swick (167); Leblond, Everett & Simmons (168)].

The manner in which protein is degraded as a result of the operation of turnover is still far from clear. It is uncertain whether proteolytic enzymes play a significant role in the process or whether degradation involves reversal of all or part of the synthetic mechanism. Mandelstam (158) has found that agents which inhibit protein synthesis in *E. coli*, such as chloramphenicol, affect degradation much less and then only after an appreciable delay. Several workers have found that agents which affect energy-yielding processes reduce degradation [e.g. see Halvorson (156); Simpson (169); Steinberg & Vaughan (170)]. Korner & Tarver (15) studied the breakdown under cell-free conditions of the protein of subcellular fractions from rat liver previously labelled *in vivo*. As these authors emphasize, interpretation of all studies on the mechanism of protein breakdown is difficult.

Virtually all the recent studies on the breakdown of labelled serum

proteins favour the view that the molecules are broken down completely
to amino acids. Thus, residues of different amino acids or of the same amino
acid incorporated at different periods of time are lost from a given protein
at the same rate as each other [e.g. see Goldsworthy & Volwiler (171);
Penn, Mandeles & Anker (172); Taliaferro (173); Walter & Haurowitz
(174); Walter *et al.* (175)]. Wiggans, Burr & Rumsfeld (176) reported,
however, that ^{35}S activity was lost more slowly than ^{14}C activity when al-
bumin labelled with ^{35}S-methionine and ^{14}C-valine was administered to rats.
The differences found were small except in one case where it was admitted
that the accuracy of measurement of the relative activity of ^{35}S and ^{14}C
was low because of a small ^{14}C content. The replicate values in this and an-
other experiment were not in good agreement. Walter *et al.* (175) did find
some unequal loss after prolonged periods of circulation of doubly-labelled
albumin; in this case unequal reutilization of the labelled moieties might be
responsible. Wiggans *et al.* suggested that their results might indicate the
existence of exchange reactions, i.e., the replacement of a given amino acid
residue of a protein without the breakdown and resynthesis of the molecule
as a whole.

    The existence of exchange reactions has been proposed as the explana-
tion for a number of experimental findings. The best evidence for their oc-
currence was provided by the work of Gale & Folkes, who showed that cer-
tain amino acids, particularly glutamic acid, could be incorporated into the
TCA-insoluble material of intact (177) or disrupted (178) *S. aureus* when
no other amino acids were added to the medium. The residues so in-
corporated could be removed from disrupted cell preparations under ap-
propriate conditions. However, more recent findings have caused this work
to be reinterpreted. Gale, Shepherd & Folkes (58) have found that two
main processes appear to occur during the incorporation of glutamate in
the absence of other added amino acids: (*a*) Attachment of glutamate resi-
dues to nonprotein components (possibly nucleotide-containing compounds).
Label is lost from these compounds in disrupted cell preparations under
conditions favouring loss of glutamate. Therefore, the suggestion that in-
corporation can occur by exchange reactions involving preformed protein
is no longer tenable. (*b*) Incorporation of glutamate residues into cell wall
material, particularly in the intact cell. The cell wall material of *S. aureus,*
which is insoluble in hot TCA, contains a number of amino acids including
glutamic acid. These amino acids can be incorporated into cell wall in the
absence of other amino acids and in the presence of chloramphenicol which
inhibits protein synthesis [Hancock & Park (179); Mandelstam & Rogers
(180)]. Gale *et al.* found that 60 per cent or more of the glutamate in-
corporated into the hot TCA-insoluble material of intact cells was present
in the cell wall fraction, but in disrupted preparations the proportion of
label present in the cell wall was much less. The significance of incorpora-
tion into noncell-wall material under these conditions is not yet clear. Direct
attempts to show the existence of exchange reactions in other systems have

not been successful, and data obtained in other studies which might indicate the occurrence of exchange can all be interpreted in other ways. The available evidence therefore does not favour the existence of exchange reactions.

ARTIFACTS IN LABELLING STUDIES

Several recent papers have emphasized that isotopically labelled amino acids can be attached to proteins by reactions other than those involved in protein biosynthesis and that the possibility of artifacts must be carefully considered in labelling studies. Activated amino acids, such as amino acid adenylates, can transfer their amino acid moieties to protein under physiological conditions by nonenzymic acylation reactions [Wieland & Pfleiderer (46); Castelfranco, Moldave & Meister (181); Zioudrou, Fujii & Fruton (182)]. Wieland & Pfleiderer suggested that, following the addition of an amino acid residue to a terminal position, rearrangement might occur which would result in transfer of the added amino acid to a nonterminal position. Cornwell & Luck (183) made a study of the binding of amino acids to insulin and histones. Most of the conditions employed were not, however, physiological. They suggested that amino acids could be bound to proteins by reaction with terminal sites. Grodsky & Tarver (184) found that alanine was very firmly bound to insulin or albumin by a reaction mediated by heavy metal ions. Protein can also be extensively labelled with isotopic amino acids as a result of enzymatically catalysed transamidation reactions [Zioudrou, Fujii & Fruton (182)].

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CARBOHYDRATE METABOLISM[1,2]

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Introduction

Because of limitations of space the reviewer has reported in some detail only on the enzymatic regulation of carbohydrate metabolism since this subject has increasingly gained in interest and since the author feels familiar with this field as a result of personal experience in it.

Oligo- and Polysaccharides

The finding of Cori *et al.* (1, 2) that skeletal muscle phosphorylase-*a* contains pyridoxal-5-phosphate has aroused new interest in this enzyme. The crystallization of phosphorylase-*b* from rabbit muscle in the presence of magnesium ions and AMP has been described by Fischer & Krebs (3). The presence of pyridoxal phosphate in this preparation was confirmed (4), and this coenzyme was demonstrated as likely to occur as a substituted aldamine, representing the reversible addition of a group across the imine double bond of a Schiff-base structure. Furthermore, in studies on the change of absorption spectra and by analysis of molecular fragments after treatment with NaBH₄, Fischer *et al.* (5, 6) presented evidence in support of the idea that pyridoxal phosphate is linked to the *ε*-amino group of lysine.

Krebs *et al.* (7) have studied in detail the conversion of crystalline phosphorylase-*b* from skeletal muscle to phosphorylase-*a* by means of a specific kinase from muscle. In this reaction the enzyme is dimerized and phosphorylated according to the following equation:

$$2 \text{ phosphorylase-}b + 4 \text{ ATP} \rightarrow \text{phosphorylase-}a + 4 \text{ ADP} \qquad (1)$$

No evidence was found as to the reversibility of the reaction. In contrast, a dimerization of the liver phosphorylase-*b* does not take place with the kinase from liver, though it too causes a conversion to phosphorylase-*a*. Likewise, the phosphorylases-*a* and -*b* from lobster muscle do not have

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different molecular weights, according to Cowgill (8). Stetten *et al.* (9) have succeeded in demonstrating a characteristic difference in substrate specificity of muscle and liver phosphorylases which renders comprehensible previous investigations on the kinetics of glycogen synthesis: the enzyme from muscle preferentially catalyzes the incorporation of glucose-1-phosphate into larger glycogen molecules, while the liver enzyme prefers smaller glycogen molecules. This can be demonstrated *in vitro* by using unfractionated samples of glycogen of different molecular size as substrate, then fractionating them for analysis after the incubation with phosphorylase. With glycogen that has been "denatured" by previous fractionation this effect is no longer found.

The dependence of glycogen content on phosphorylase activity has been shown by Brody (10) with the myometrium of pregnancy: changes in the phosphorylase activity are accompanied by parallel alterations in the amount of glycogen. In addition, in seven different ascites tumors it has been demonstrated (11) that the low content of glycogen (perhaps, there is no glycogen at all) has to be ascribed to an extremely small phosphorylase activity, amounting to only 1/10 to 1/20 of mouse liver activity.

Leloir & Cardini (12) characterized an enzyme from the soluble fraction of liver that incorporates UDP-bound glucose into glycogen in the presence of glycogen or soluble starch as a primer. This corroborates the hypothesis of Niemeyer (13) that phosphorylase and amylase are predominantly responsible for glycogen breakdown, whereas the synthesis is uridine linked.

In pancreatic juice of the rat, the concentration of α-amylase is often so high that the enzyme crystallizes spontaneously (14).

Cellulose metabolism has been investigated in a variety of molds and bacteria [(15 to 23); for a summary see (24)]. According to Hash & King (19, 20), various β-glucosidases are present in the fungus *Myrothecium verrucaria,* the specificity of which is dependent on the chain length of the substrate. Cellobiose was not found to be an obligatory precursor of glucose in cellulose breakdown. "Cellulose dextrins" (hexasaccharides, etc.) may be formed from cellobiose by transglucosidation. High-speed-centrifuged particles from *Acetobacter xylinum* were shown by Glaser (21) to contain an enzyme which produces cellulose from UDP-glucose. Similar to glycogen synthesis from UDP-glucose a primer is required, such as cellodextrins.

In various grasses, structure, synthesis, and breakdown of polyfructosans have been investigated in their dependence upon various, particularly seasonal conditions by Schlubach (25). Avigad & Feingold (26) investigated the fructosides produced from sucrose by a corynebacterium.

For the synthesis of hyaluronic acid in streptococci, it has been reported by Markovitz *et al.* (27) that glucose is a precursor of the glucuronic acid and glucosamine moieties. Tritiated UDP-glucuronic acid together with UDP-N-acetyl glucosamine, ATP, and N-acetyl glucosamine-1-phosphate were incubated with strain A streptococci and found to produce appreciable

amounts of hyaluronic acid. This indicates that the synthesis of hyaluronic acid is very likely to occur via the UDP compounds. The occurrence in group A streptococci of the UDP compounds mentioned has been ascertained chromatographically (28).

Glaser & Brown (29) reported that the formation of chitin in a cell-free preparation from *Neurospora crassa* utilizes UDP-N-acetyl glucosamine, and requires an acetyl glucosamine polymer as a primer:

$$\text{UDP-acetyl glucosamine} + (\text{acetyl glucosamine})_n \rightleftharpoons \text{UDP} + (\text{acetyl glucosamine})_{n+1} \quad (2)$$

The decomposition of chitin has been investigated by Berger & Reynolds (30) with a chitinase from *Streptomyces griseus*. This enzyme hydrolyzes chitin to N-acetyl glucosamine and N,N-diacetyl chitobiose without forming higher saccharides. Besides, an enzyme could be demonstrated in this organism hydrolyzing the di- and trisaccharides of N-acetyl glucosamine, but none of the higher saccharides.

Maltose, maltotriose, and maltotetraose have been demonstrated in liver by Fishman *et al.* (31). That the amount of these compounds fluctuated according to the glycogen level in starvation and on the action of insulin suggested their participation in glycogen synthesis (32). In this connection, *trans*-α-glucosylase from bovine plasma is of interest; it was investigated in detail by Miller & Copeland (33) and by Miller (34). The enzyme synthesizes by transglucosylation maltotriose and other oligosaccharides from maltose in the presence of Ca (or Sr) ions.

In yeast cells trehalose, in addition to glycogen, is part of the reserve carbohydrates. Cabib & Leloir (35) purified an enzyme (already previously demonstrated by these authors) 20-fold from brewer's yeast, catalyzing the reaction:

$$\text{UDP-glucose} + \text{glucose-6-phosphate} \rightarrow \text{UDP} + \text{trehalose phosphate} \quad (3)$$
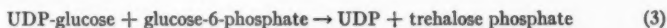
Reversibility of the reaction could not be demonstrated. In yeast the liberation of trehalose from trehalose phosphate is catalyzed by a specific phosphatase activated by Mg^{++} (35). From larvae of the wax moth, *Galleria mellonella*, Kalf & Rieder (36) have purified a trehalose which has been proposed for use in the assay of trehalose because of its good affinity toward this disaccharide ($K_m = 1.3 \times 10^{-4}$ M). This enzyme claims interest in connection with the function of trehalose as the "blood sugar" of insects.

Avigad (37) has demonstrated that levan sucrase from *Aerobacter levanicum* forms a new trisaccharide, α-lactosyl-β-fructofuranoside, from sucrose and lactose by the transfer of a β-fructofuranosyl residue. The specificity of the enzyme has been characterized by the use of various sugars as acceptors for the fructosyl residue (38). Weidenhagen & Lorenz (39) have identified α-glucosido-6-fructofuranose as a reaction product of sucrose after incubation with an extract of dried entero bacteria, the yield is 80 to 90 per cent.

Experiments of Preiss (40) on the inactivation of invertase in yeast cells by means of low voltage electrons resulted in accord with previous studies of other authors in localizing the enzyme at the surface of yeast cells (between 500 Å and 1000 Å depth).

With an extract from mammary tissue, containing galactosyl transferase, Gander et al. (41) demonstrated in detail the synthesis of lactose phosphate, already previously outlined (42) according to the equation:

$$\text{UDP-galactose} + \text{glucose-1-phosphate} \rightarrow \text{lactose-1-phosphate} + \text{UDP} \qquad (4)$$

Isotope experiments by Wood et al. (43) suggest, however, that additional mechanisms are involved in lactose formation with mammals (44).

Wallenfels & Zarnitz (45) report on a β-galactosidase crystallized from Escherichia coli, ML 309. There is no difference in the ratio of transglycosidizing to hydrolyzing activity of the crystalline enzyme and the crude extract. Therefore, hydrolyzing and transferring activity are doubtlessly linked to one single enzyme. The properties of the enzyme were compared to the corresponding β-galactosidase from calf intestine with respect to substrate specificity and metal ion requirements (46). Hu & Reithel (46b) also reported on the purification and crystallization of bacterial β-galactosidase. This enzyme, isolated from E. coli, ML 308, requires Mg++ in contrast to the enzyme obtained by Wallenfels & Zarnitz (45) from E. coli, ML 309. Pazur et al. (47) have shown with an enzyme preparation from Saccharomyces fragilis that transgalactosylations are reversible processes. New oligosaccharides were synthesized by enzymatic transfer of the galactosyl unit of lactose as substrate to sucrose, planteose, raffinose, or glucosamine as cosubstrates (48).

GLYCOLYSIS, ALCOHOLIC FERMENTATION, AND GLUCONEOGENESIS

The mechanism of action of hexokinase has been investigated in some detail with crystalline yeast hexokinase. On the basis of analyses of the metachromatic reaction concurrently with the concentration of orthophosphate, it was presumed that metaphosphate may act as an intermediate in the hexokinase reaction (49). Hudson & Woodward (50) succeeded in attributing glucosone inhibition of anaerobic and aerobic glucose breakdown in yeast and of tissue slice glycolysis to an inhibition of hexokinase. In yeast glucosone caused a corresponding inhibition of aerobic and anaerobic fermentation, but did not affect respiration. Presumably, pyruvate, the formation of which is reduced after hexokinase inhibition, is still oxidized at the same rate as before because of the low Michaelis constant of pyruvate oxidase toward pyruvate ($K_m = 10^{-4}$M); while pyruvate decarboxylase, which is required for the production of alcohol, i.e., for aerobic fermentation, operates at a diminished rate because of its lower affinity for pyruvate ($K_m = 10^{-3}$M) (51).

The phosphate which is bound to rabbit muscle phosphoglucomutase and

involved in the phosphate transfer (52) is probably esterified with serine (53, 54). According to Wosilait (55), the same is true of the phosphate bound to liver phosphorylase which is necessary for the activity of the enzyme. Phosphoglucose isomerase has been purified from erythrocytes by Tsuboi *et al.* (56). Nirenberg & Hogg (57) have reported that the inhibition of glycolysis in Ehrlich ascites tumor cells by 2-deoxy-D-glucose is accounted for by the phosphorylation of this sugar to the 6-phosphate and subsequent inhibition of phosphoglucose isomerase by the phosphorylated sugar.

Spectroscopic measurements and studies with deuterium have led to the result that dihydroxyacetone phosphate, contrary to phosphoglyceraldehyde, is activated in the condensing reaction catalyzed by yeast and muscle aldolase. Further experiments by Rose (58) with crystalline aldolase indicate an exchange reaction between C-4,5,6 of fructose-1,6-diphosphate and glyceraldehyde-3-phosphate. Rose assumed that this finding might explain the asymmetric labelling of glucose observed in photosynthesis experiments by Gibbs & Kandler (59).

The reversible hydrogenation of acetol phosphate to 1,2-propanediol-1-phosphate has been described by Sellinger & Miller (60) as occurring with glycerophosphate dehydrogenase. The Michaelis constant of the enzyme with dihydroxyacetone phosphate is 330 times lower than it is with acetol phosphate. The authors (61), however, suppose that in rabbit muscle there occurs in addition to α-glycerophosphate dehydrogenase another enzyme which reversibly dehydrogenates propanediolphosphate with DPN. Since, in contrast to commercial yeast alcohol dehydrogenase preparations, commercial crystalline α-glycerophosphate dehydrogenase from rabbit muscle specifically reacts with DPN, without any utilization of TPN, this enzyme may be used to assay DPN or DPNH without interference by TPN and TPNH respectively (62).

The properties of the bound DPN of muscle triosephosphate dehydrogenase have been studied by Kaplan *et al.* (63, 64). These authors conclude from the interaction of bound DPN with various dehydrogenases, DPNase, adenosine deaminase, DPN kinase, and snake venom pyrophosphatase, as well as from experiments on DPN analogues, that the binding of DPN to the protein is effected through at least two linkages, one of which will probably involve the nicotinamide moiety. The ability of muscle and yeast triosephosphate dehydrogenase to transfer acyl moieties to the SH group of methyl mercaptan has been demonstrated by Wolff (65). The acyl moiety of 1,3-diphosphoglyceric acid produced by the dehydrogenation of phosphoglyceraldehyde is transferred to the thiol group of methyl mercaptan forming phosphoglyceryl methylthiol ester. The synthesis of the methylthiol ester is analogous to the formation of acetyl-S-coenzyme A and acetyl-S-glutathione in the dehydrogenation of acetaldehyde by triosephosphate dehydrogenase, which was previously described by Harting & Velick (66).

As is well known, 2,3-diphosphoglycerate, the coenzyme of phospho-

glycerate mutase, is present in high concentrations in red blood cells. Determination of the concentration of this compound as well as other phosphorylated intermediates of glycolysis in erythrocytes have been performed by a variety of authors (67, 68, 69). Diphosphoglycerate mutase, which is required for the formation of 2,3-diphosphoglycerate from 1,3-diphosphoglycerate, is present with high activity in chicken breast muscle. Grisolia & Joyce (70) take advantage of this fact for the preparation of 2,3-diphosphoglycerate with such an extract according to the over-all equation:

$$\text{2,3-phosphoglycerate} \rightarrow \text{2,3-diphosphoglycerate} + \text{pyruvate} \qquad (5$$
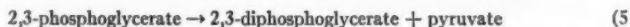
Glycerate-2,3-diphosphatase has been purified from baker's yeast and chicken breast muscle by Joyce & Grisolia (71). With respect to kinetic data some differences have been found in comparison with the enzyme previously characterized by Rapoport *et al.* in rabbit and rat muscle. The activation of the enzyme by Hg has been ascribed by Sauer (72) to the activity of Hg complexes with amino acids and proteins.

The equilibrium constant of the enolase reaction has been determined by Wold & Ballou (73), taking into consideration the metal-binding constants of the substrates. In addition, the reaction kinetics of the purified enzyme have been investigated (74, 75, 76). Grisolia *et al.* (77) have reported a new assay method for pyruvate kinase based on the colorimetric determination of pyruvate which is produced from 3-phosphoglyceric acid after the addition of auxiliary enzymes. Since ADP and ATP are involved catalytically in the reaction series, these nucleotides, too, can be estimated by this test in very small concentrations. McQuate (78) determined the equilibrium constant at various pH values as well as the Michaelis constants of the pyruvate kinase reaction by means of the crystalline enzyme from rabbit muscle.

Animal lactic dehydrogenase now available from various organs in pure state has been intensively studied. Winer & Schwert (79) described quantitatively the influence of pH on the kinetics of lactate dehydrogenation, assuming a lactate-DPN- and a lactate-DPNH-protein complex respectively in which an SH group is involved. A lactic dehydrogenase-DPNH complex could be demonstrated by its characteristic fluorescence spectrum (80). The characteristic fluorescence of the complex may be used to estimate the binding capacity of the protein for DPNH: four moles of DPNH were shown to be bound to one mole of protein (81). According to Shifrin & Kaplan (82), acetyl pyridine-DPNH, an analogue of DPNH, forms a characteristically fluorescent complex with lactic dehydrogenase. Pfleiderer *et al.* (84) have reported that the addition of DPN-sulfite and DPNH plus lactate respectively to lactic dehydrogenase creates a protection from denaturation by heat or urea, since these substances are very firmly bound to those areas of the lactic dehydrogenase surface which are modified by thermal denaturation causing loss of activity. Presumably the substances in question are bound to SH groups of the enzyme. Moreover, Pfleiderer

et al. (83) have studied the content of zinc in lactic dehydrogenases of different origin. After the treatment with agents forming Zn complexes, the authors showed the enzymes to have retained all the activity but no zinc. Accordingly, Zn is not likely to participate in the catalytic action of the enzyme.

By means of the electrophoretic separation of enzyme extracts from various organs, Wieland, Pfleiderer, and Jeckel (85, 86) demonstrated that in the same organ there may occur protein fractions differing in their electrophoretic behaviour but all showing lactic dehydrogenase activity. In experiments with a large variety of vertebrates (fish, amphibians, reptiles, birds, various mammalians, etc.) Haupt & Giersberg (87) extended still further the findings of Pfleiderer *et al.* and demonstrated in detail the heterogeneity of lactic dehydrogenases in organs.

While normally dextrorotatory L-lactic acid is produced as the final product of glycolysis, Warburg *et al.* (88) have shown chlorella to accumulate levorotatory D-lactic acid under anaerobic conditions. *In vitro* pyruvate could be hydrogenated to D-lactic acid with a chlorella extract. To identify the antipodes, the zinc salt of lactic acid was isolated and submitted to polarimetric measurement.

Lactate-dehydrogenating cytochrome enzyme from yeast has been used by Wieland (89) to determine lactic acid spectrophotometrically. Concerning the mechanism of action of the yeast enzyme, Marcus & Vennesland (90) have demonstrated the keto form of pyruvic acid to be the first reaction product of lactate dehydrogenation, as was previously found for the animal DPN-dependent enzyme.

In several plants the occurrence of pyruvic decarboxylase required for alcoholic fermentation has been demonstrated (91, 92). Betz (93, 94) has shown that the extra carbon dioxide (i.e., CO_2 exceeding a respiratory quotient of one) formed under aerobic conditions in root tips, results from alcoholic fermentation, since an accumulation of alcohol equivalent to the extra carbon dioxide produced is observed. According to Hatch & Turner (95), starch and hexoses are fermented to alcohol by extracts from pea seeds. The authors did not find that alternate pathways of hexose degradation operated to any significant extent.

Ebisuzaki & Barron (96) separated in yeast a new second alcohol dehydrogenase from the alcohol dehydrogenase known for some time. The new enzyme differs from the classical one particularly by another spectrum of activity with various alcohols.

It has been demonstrated by Zebe *et al.* (97) that in insect muscles under anaerobic conditions a mode of glucose breakdown takes place similar to the so-called second form of fermentation described by Neuberg as occurring in yeast cells under certain conditions. The terminal products of this fermentation in insect muscle are α-glycerophosphate and pyruvate. This finding has since been confirmed and extended by a variety of authors (98, 99, 100, 431, 432). This type of fermentation is caused by a lack of

DPN-dependent lactic dehydrogenase in insect muscles. Therefore, pyruvate, the product of fermentation, cannot be converted further, and the hydrogen, supplied by triosephosphate dehydrogenation, is utilized to hydrogenate dihydroxyacetone phosphate to α-glycerophosphate. This reaction is catalyzed by α-glycerophosphate dehydrogenase, which in insect muscle is present with high activity (97, 101). During the aerobic phase an oxidation to CO_2 and H_2O of the accumulated products of fermentation takes place. In intact American cockroaches, glucose is catabolized by way of the citric acid cycle while the pentose phosphate cycle is not markedly involved (102). High respiratory activity of insect muscle homogenates with α-glycerophosphate was reported by Zebe (104, 105) as early as 1956. Sacktor et al. (106) have demonstrated P/O ratios of 1 to 2 when α-glycerophosphate is oxidized by insect muscle sarcosomes. α-Glycerophosphate oxidase from flight muscle mitochondria, which starts this oxidation, has been characterized by Sacktor & Estabrook (107) with respect to its Michaelis constant with glycerophosphate ($K_m = 2 \times 10^{-3}$ M), pH optimum, etc. α-Glycerophosphate oxidase from yeast granules and mammalian mitochondria (brain and muscle) was extracted by Ringler & Singer (108) with digitonin. Phenazine methosulfate was shown to be the best electron acceptor for activity measurements (108, 109). In contrast to the α-glycerophosphate oxidase localized in mitochondria, the DPN-dependent enzyme is present exclusively in the particle-free supernatant from muscle, according to Young & Pace (110). Liver and kidney do not show appreciable activities.

Mendicino & Utter (111) have demonstrated gluconeogenesis in vitro with a reconstructed system using lactate and malate as substrates. After the addition of mitochondria and glycolytic enzymes together with fructose diphosphatase, the synthesis of hexose monophosphates could be shown. The formation is promoted by DPNH, formed by lactate dehydrogenation, by adenosine triphosphate, originating in mitochondria, as well as by phosphoenolpyruvate produced in mitochondria from malate. For a long time by-pass of the pyruvate kinase reaction was taken to be indispensable for gluconeogenesis from pyruvate. Tracer experiments by Hiatt et al. (112), however, have furnished evidence that this mechanism of gluconeogenesis via "dicarboxylic acid shuttle" plays a role in liver only, while in muscle the formation of glucose takes place by a direct reversal of the pyruvate kinase reaction. These conclusions were drawn from the observation that in liver, with C_2-labelled pyruvate, a randomization between C-1 and C-2 and between C-5 and C-6 respectively of the hexose produced is caused by the "dicarboxylic acid shuttle," whereas in muscle no or only insignificant randomization is observed. Since the conversion of pyruvate via malate and oxaloacetate to phosphoenolpyruvate depends on the amount of TPNH available for the action of malic enzyme, the difference between the two tissues (113) may rest on the TPNH concentration, which in muscle amounts to only 1/10 as compared to liver (114). In contrast to hitherto existing belief, it is supposed by Racker (115) that fructose diphosphatase

may not play an important role in gluconeogenesis because of the poor activity of this enzyme in physiological pH ranges. It is furthermore suggested by Racker that the enzymes of the pentose phosphate cycle may play a role in gluconeogenesis. The sedoheptulose diphosphatase, demonstrated by Racker & Schroeder (116) in spinach, yeast, and animal tissues, occupies a key position by shifting the reaction sequence toward hexose synthesis.

CITRIC ACID CYCLE AND GLYOXYLATE CYCLE

During recent years, several oxidative pathways of carbohydrate degradation have been described which may operate in addition to the citric acid cycle. Particular interest has been given to the questions: in which organisms and under which physiological conditions is involved the citric acid cycle in biological oxidations. Experiments of Stoppani *et al.* (117) with yeast cells which had been administered acetate-^{14}C support the hypothesis that by virtue of ^{14}C distribution in succinic acid the citric acid cycle operates in yeast. Since carbon dioxide fixation in yeast predominantly proceeds via the formation of oxaloacetate, Stoppani *et al.* (118) suppose the glyoxylate cycle to be of little if any importance as compared to the citric acid cycle. Experiments of Eaton & Klein (119) also suggest that the citric acid cycle plays a decisive role in the oxidation of glucose by yeast. Studies by Hill *et al.* (120) in which ^{14}C-labelled acetate, pyruvate, and glycerol were injected intraperitoneally to rats, suggest according to the labelling pattern of glutamic acid, aspartic acid, alanine, and serine an operation of the citric acid cycle. However, in all experiments in which radioactive substrates were applied, it must be kept in mind that the evidence obtained is not absolutely conclusive as to the entry of a substrate into a certain metabolic pathway. For example (121, 122), the false conclusions drawn from tracer experiments, before Ogston had established his three-point theory, ought to be remembered. According to these conclusions, iso-citric acid was said to be the first condensation product of the citric acid cycle.

Meadow & Clarke (123) have shown that in *Pseudomonas aeruginosa* the citric acid cycle probably operates. The lag periods preceding the oxidation of certain intermediates of the citric acid cycle are thought by the authors to be caused by the adaptive formation of "permeases," which are said to mediate the transport of substrates across the cell wall, according to Cohen & Monod (124). In the leaves of various leguminous plants Soldatenkov & Mazurova (125) have shown malonic acid to be present in amounts exceeding that which would inhibit succinic acid dehydrogenation, thus rendering unlikely the operation of the citric acid cycle in these plants. In erythrocytes, Dajani & Orten (126) have shown by the use of column chromatographic determinations of the concentration of intermediates that in nucleated, respiring red blood cells the citric acid cycle is active, but is inactive in non-nucleated erythrocytes. In this connection Wagenknecht & Rapoport (127) have shown that an inhibitor of succinate dehydrogenation is present in reticulocytes. Tissières *et al.* (128) have isolated particles

from *Azotobacter vinelandii* catalyzing respiratory chain phosphorylation but not oxidizing α-ketoglutarate and fumarate respectively. The oxidation of these substrates does not proceed prior to the addition of cytoplasm. Therefore, in these bacteria, in contrast to animal cells, respiratory chain phosphorylation and citric acid cycle enzymes are not localized in the same particles.

Oxidation of pyruvate and α-ketoglutarate.—According to Holzer & Goedde (129), yeast decarboxylase is able to oxidize pyruvate in the presence of a suitable hydrogen acceptor, namely 2,6-dichlorophenolindophenol. Disappearance of pyruvate and reduction of the indophenol were shown to be accompanied by a stoichiometric formation of acetate. This finding with yeast carboxylase is in support of the assumption that the intermediate in anaerobic and aerobic pyruvate conversion is the same, namely "activated acetaldehyde." Besides pyruvate decarboxylase, which has long been recognized, Holzer & Goedde (51) have demonstrated that in yeast a pyruvate oxidase exists with essentially the same properties as the known animal and bacterial pyruvate oxidases. The oxidase is localized in yeast mitochondria and may be solubilized by acetone treatment (51). The occurrence of this enzyme in yeast mitochondria has been confirmed by Alvarez *et al.* (130). Goldman (131, 132) has demonstrated in *Mycobacterium tuberculosis* a pyruvate oxidase which can be assayed by means of dichlorophenolindophenol, ferricyanide or, manometrically, by O_2 consumption. The oxidase requires thiamine pyrophosphate, DPN, and coenzyme A. Acetyl coenzyme A has been shown to be the end product of oxidation. Lipoic acid probably participates in pyruvate oxidation by this system, since a DPN-specific lipoic dehydrogenase activity could be detected in the oxidase preparation.

Concerning the question, if thiamine pyrophosphate is involved in pyruvate oxidation as a redox system (in this case a transition from the disulfide to the thiol compound was postulated), Engelhardt & Kanopkaite (133) could show that neither pyruvate decarboxylase from yeast nor pyruvate oxidase from pigeon breast muscle are activated by the disulfide form of thiamine pyrophosphate. Therefore, thiamine pyrophosphate probably does not take part in the redox process, but is rather involved in the first stage of anaerobic and aerobic decarboxylation by the formation of "activated acetaldehyde."

By means of a pyruvate oxidase system from lipoic acid-deficient *Streptococcus faecalis*, Reed *et al.* (134) showed that protein-bound lipoic acid rather than free lipoic acid is required for pyruvate oxidation. ATP, a divalent metal ion, and a specific activating protein fraction (which can be separated from oxidase activity) are required for the activation of lipoic acid by means of binding to the protein. Synthetic lipoyl adenylate has been shown to replace lipoic acid and ATP in the activating process. It appears, therefore, that the activation consists of the formation of a "lipoyl enzyme." In addition, an enzyme fraction was obtained by Reed *et al.* (135) which inactivated pyruvate oxidation systems prepared from *E. coli* and *S. faecalis*

and released α-lipoic acid. Thus, the hypothetic "lipoyl enzyme" is likely to be hydrolyzed. According to Reed *et al.* (135), the reduction of free non-protein-bound lipoic acid in pyruvate oxidation, observed by various authors, appears to be mediated through protein-bound lipoic acid. Presumably, a "disulfide interchange" reaction between free disulfide lipoic acid and protein-bound dihydrolipoic acid takes place. Sanadi *et al.* (136) also conclude from experiments on the arsenite inhibition of mammalian α-keto-glutaric dehydrogenase that the disulfide form of lipoic acid is acylated only when bound to the enzyme, and that only enzyme-bound succinyl lipoate transfers the succinyl group to coenzyme A. According to Sanadi *et al.* (136, 137), thiol transacetylase, which catalyzes a direct transfer of acyl from free lipoic acid to coenzyme A (138), has nothing to do with α-ketoglutarate and pyruvate oxidation.

Goldman (139) has resolved a soluble pyruvate oxidase from *M. tuberculosis* into different fractions, one of which catalyzes the sequence of steps leading to the formation of acetyl coenzyme A. The reversible dehydrogenation of lipoic acid with DPN is catalyzed by both the first fraction and another separable fraction. For further elucidation of the enzymatic steps of pyruvate oxidation an acetate-requiring mutant of *E. coli* has been studied in detail by Gounaris & Hager (140, 141). This mutant fails to oxidize pyruvate to acetyl coenzyme A, although the two steps accessible to assay, i.e., lipoic transacetylase as well as lipoic dehydrogenase, are present. It is, therefore, suggested that the genetic block lies in the first reaction steps, leading from pyruvate to acetyl-S-lipoic acid. After the mutant extract is combined with other pyruvate oxidase preparations, the first two reaction steps may be tested (140). An interesting effect of α-lipoic acid has been observed by Lettré (142): the injection of α-lipoic acid considerably increases growth and mitotic rate of ascites tumors.

In *Mycobacterium phlei* a second lactate-oxidizing enzyme has been detected by Sutton (143), in addition to "lactic oxidative decarboxylase" which had already been crystallized (144). Both enzymes oxidize lactate to acetate without passing the pyruvate stage. In contrast to the enzyme described earlier, the new one will utilize Neotetrazolium as an electron acceptor. To restore the activity of the new enzyme after dialysis an activator still unknown is required. The two enzymes in *M. phlei* appear to compete for the utilization of lactate.

Acetoin formation.—The mechanism of acetoin production has been investigated in detail by Dawson *et al.* (145) with a pyruvate oxidase preparation from pig heart. In accordance with previous studies, the authors suggest that active acetaldehyde formed from pyruvate is condensed with free acetaldehyde to form acetoin. In the presence of an excess of pyruvate, however, α-acetyl lactic acid is probably the first product to be formed. Taylor & Juni (146) have shown that a variety of bacterial enzymes exist which stereospecifically reduce D- and L-acetoin to the corresponding butanediols, DPN being required as a coenzyme. Strassman *et al.* (147, 148) have

demonstrated α-acetolactate besides acetoin to be produced from pyruvate in yeast. With TPN, α-ketoisovaleric acid is formed from α-acetolactate. By transamination α-ketoisovaleric acid may be converted to valine.

Enzymes of citric acid cycle.—Grant & Mongkolkul (149) have shown that in adrenocortical mitochondria the DPN-linked isocitric dehydrogenase has higher activity than the TPN-linked enzyme. In these particles only a low activity of the nucleotide transhydrogenase could be observed. According to Ernster & Navazio (150), in rat liver the DPN-specific isocitric dehydrogenase is exclusively localized in the mitochondria, while the TPN-specific dehydrogenase is distributed between the mitochondria and the soluble fraction in an approximate proportion of 1:9. The quantitative participation of the DPN-linked enzyme in isocitrate oxidation is estimated to be 75 per cent.

γ-Amino butyric acid formed in brain by glutamate decarboxylation enters the citric acid cycle on the succinate level. Baxter & Roberts (151) have investigated a transaminase from brain tissue transaminating γ-amino butyric acid with α-ketoglutarate to succinic semialdehyde and glutamic acid. By means of DPN, the succinic semialdehyde is dehydrogenated to succinate. This dehydrogenase has been purified 150-fold from monkey brain by Albers & Salvador (152). An enzyme catalyzing the same reaction preferentially with TPN was enriched from pseudomonas by Scott & Jakoby (153). The reaction is not reversible. Singer *et al.* (154-160) have studied in detail the succinic dehydrogenase from yeast, bacteria, and ox heart. The enzyme from yeast and animal tissue could be shown to operate reversibly. This finding (155), together with other observations, suggests the identity of this enzyme with fumaric reductase previously described by F. G. Fischer. From the obligate anaerobe *Micrococcus lactilyticus,* too, succinic dehydrogenase was isolated (156). The enzyme represents an iron-flavin adenine dinucleotide protein but differs from the succinic dehydrogenase in animal tissues and in yeast in catalyzing the reduction of fumarate much more rapidly than the oxidation of succinate (157).

A malate-oxidizing enzyme has been demonstrated by Cohn (161, 162). It does not require DPN and can be tested only by means of ferricyanide as an electron acceptor. The reaction product, oxaloacetate, is characterized by way of its reducibility by DPN-linked malic dehydrogenase. Possibly the enzymatic activity oxidizing malate with ferricyanide is an unphysiological one, such as the oxidation of pyruvate to acetic acid by pyruvate decarboxylase from yeast with dichlorophenolindophenol as an electron acceptor (129).

Carboxylation of pyruvate and phosphoenolpyruvate.—The maintenance of citric acid cycle operation requires a supply of four carbon dicarboxylic acids. In as much as carbohydrate metabolism is involved, a carboxylation of pyruvate and phosphoenolpyruvate takes place. Freedman & Graff (163) have shown that in rats the distribution of pyruvate to the carboxylation to four carbon dicarboxylic acids on the one hand, and to the oxidation to acetyl coenzyme A on the other, is dependent on the state of nutrition. In

the fasted animal pyruvate is predominantly carboxylated. Malic enzyme, which was first shown by Ochoa *et al.* to carboxylate pyruvate by means of TPNH, has been purified 1000-fold from pigeon liver by Rutter & Lardy (164). After cell fractionation, the enzyme is quantitatively recovered from the supernatant fraction. The enzyme described by Bandurski *et al.*, which irreversibly carboxylates phosphoenolpyruvate to oxaloacetate, has been characterized in extracts from crassulacean plants by Walker *et al.* (165, 166) and shown to occur in *Thiobacillus thiooxidans* by Suzuki & Werkman (167, 168). This is different from Utter's enzyme, which is inosine diphosphate-dependent and operates reversibly. The enzyme from *T. thiooxidans* is distinguished by its high affinity for bicarbonate ($K_m = 1.2 \times 10^{-3}$ M). It is probably responsible for CO_2 fixation in aspartate via oxaloacetate by means of colorless chloroplast extracts from spinach, as observed by Rosenberg *et al.* (169).

Glyoxylate cycle and other $C_2 \rightarrow C_4$ mechanisms.—The reaction chain called glyoxylate cycle, which was formulated first by Kornberg & Krebs (170), catalyzes the conversion of two moles of acetic acid to succinic acid. Whether there exists, in addition to the glyoxylate cycle, another enzyme system catalyzing the dehydrogenation of two moles of acetic acid to succinic acid has been studied with contradictory results by a variety of authors. Since, according to Madsen (171), the key enzymes of the glyoxylate cycle, isocitritase and malate synthetase, do not occur in animal tissues, another mechanism must account for the conversion of fat to carbohydrates which probably does exist [see for example (172)]. Seaman (173) has repeated and extended his former findings on an enzyme system reversibly converting succinate to acetyl coenzyme A in rat brain, heart, liver, and skeletal muscle. The enzyme, which requires DPN, was purified 120-fold from tetrahymena. The rate of acetyl coenzyme A dehydrogenation to succinic acid is only about one tenth as rapid as is the rate of succinate cleavage. The equilibrium is markedly in favor of acetyl coenzyme A. Davies (174) presents evidence that an enzyme system occurring in pig heart catalyzes the dehydrogenation of acetate to form succinate. Coenzyme A as well as DPN are required. The formation of acetyl coenzyme A from succinate by liver slices, communicated by Topper *et al.* (175), occurs in minimal amounts if at all, according to Sutherland (176).

The two pivotal enzymes required in addition to the citric acid cycle to guarantee the transition of acetic acid to succinic acid have as yet been demonstrated only in bacteria and plant tissues. Wong & Ajl (177) have purified malate synthetase 50-fold from *E. coli,* in which organism this enzyme had been demonstrated for the first time (178). The enzyme from *E. coli* hitherto could not be shown to reverse the reaction, i.e., to catalyze the cleavage of malate. Isocitritase has been isolated from *P. aeruginosa* by Smith & Gunsalus (179). The purification obtained was 30-fold. The equilibrium of the reaction is displaced toward isocitrate formation ($K = 34$). The enzyme has been demonstrated in germinating marrow seedlings by

Heydeman (180). In accordance with previous findings by Kornberg & Beevers (181), the enzyme may be involved in the conversion of fat to carbohydrates as part of the glyoxylate cycle. Kornberg *et al.* (182, 183, 184) have demonstrated *in extenso* that also in pseudomonas and corynebacterium the glyoxylate cycle participates in the conversion of acetate to four carbon dicarboxylic acids, this process being indispensable for the growth on acetate as the only carbon source. No evidence was obtained by these authors as to a direct reaction of acetate to yield succinate. According to Kornberg & Collins (185), the activities of aceto-coenzyme-A-kinase (acetyl thiokinase), malate synthetase, and isocitritase are likewise sufficient in *Aspergillus niger* to account for growth on acetate involving the glyoxylate cycle.

Bolcato *et al.* (186, 187) reported acetic acid to be oxidized via glycollic acid and glyoxylic acid to formic acid and CO_2 in yeast and *E. coli*. However, a definite enzyme catalyzing the hydroxylation of acetate to glycollate has not yet been characterized. Glycollic acid oxidase originally demonstrated by Zelitch & Ochoa (188) has been crystallized as flavin mononucleotide protein from spinach leaves by Frigerio & Harbury (189). Mothes & Wagner (190) showed that the enzyme is generally present in higher plants and that its activity depends upon the stage of development of the plant. Zelitch (191) reported that a considerable amount of respiration of leaves in light proceeds via the glycollic acid oxidase system. The reverse reaction from glyoxylic acid to glycollic acid is catalyzed by a DPN-specific enzyme isolated from tobacco leaves by Zelitch (192), as well as from spinach leaves by Holzer & Holldorf (193). Both enzymes reversibly reduce glyoxylate and hydroxypyruvate to form glycollate and D-glycerate respectively. The enzyme from tobacco, however, operates more rapidly with glyoxylate than with hydroxypyruvate and for this reason was called glyoxylate reductase by Zelitch, whereas the enzyme from spinach, designated D-glycerate dehydrogenase, reduces hydroxypyruvate three times as rapidly as glyoxylate. Presumably the DPN-dependent glycollate dehydrogenase is also involved in a dismutation of glyoxylate to glycollic acid and oxalic acid by extracts from *A. niger*, as was observed by Franke & De Boer (194). The oxidation of glyoxylate by rat liver has been studied by D'Abramo *et al.* (195), as well as by Nakada & Sund (196, 197). These authors showed that the mitochondrial enzyme system oxidizing glyoxylate to formate and CO_2 required DPN, manganese, thiamine pyrophosphate, and L-glutamate as cofactors. A condensation product from glyoxylate and glutamate is likely to be produced as intermediate.

Pentose Phosphate Pathway and Related Metabolic Processes

Thanks to the elucidation of the intermediary steps of pentose phosphate cycle, two pathways now are known which lead from hexoses to ribose: (*a*) the well-known oxidative decarboxylation of glucose-6-phosphate, and (*b*) a conversion of hexose phosphates and triose phosphates to pentose phos-

phate catalyzed by transketolase and transaldolase without involving redox processes.

Bagatell *et al.* (198) and Bernstein & Sweet (199) have shown in experiments with labelled hexoses that in *E. coli* ribose is synthesized by both pathways. The oxidation of glucose, however, is preferentially involved in ribose formation (199). In these experiments (198, 199) the labelling patterns of deoxyribose and ribose were essentially the same, suggesting synthesis of the two sugars via the same pathway. Reichard (200) presented further evidence which indicated that the conversion of ribose to deoxyribose proceeds in chicken embryos on the nucleotide level without cleavage of the glucoside bond. The details of deoxyribose synthesis are not available. Shuster & Goldin (201, 202) have shown in mouse liver that the anaerobic transketolase-transaldolase pathway can account for 90 to 95 per cent of ribose formation, whereas only 5 to 10 per cent was found to be produced by glucose oxidation. Hiatt (203) has investigated the path of ribose production from glucose in the rat by simultaneous administration of glucose-2-^{14}C and imidazole acetic acid. The ribose produced from glucose is excreted in urine as imidazole acetic acid riboside. By analysis of the isotope distribution in the ribose moiety of the excretion product, it was demonstrated that 30 to 50 per cent of glucose was converted to ribose by oxidation (C-1-decarboxylation), while the remainder is produced by the transketolase reaction. In thiamine-deficient rats Hiatt found this proportion to be strongly shifted in favor of the oxidative pathway: now 80 to 90 per cent of ribose is formed via the oxidative path, because thiamine pyrophosphate is required for transketolase activity. Brin *et al.* (204) had previously shown that in erythrocytes of thiamine-deficient rats the activity of transketolase is decreased. According to Kit *et al.* (205), pentoses are formed from hexoses in normal and malignant lymphatic cells by both the anaerobic and the aerobic pathway.

Preparation procedures and spectrophotometric tests for substrates and enzymes of the pentose phosphate pathway developed by Racker *et al.* (206, 207, 208) have intensified and advanced studies on the importance of this pathway in a great many organisms. Chefurka (209) has reported that all the enzymes of the pentose phosphate cycle are present in extracts from house flies. From experiments on the lactating cow, Black *et al.* (210) concluded that 50 to 70 per cent of glucose is metabolized by the pentose phosphate pathway. Kinoshita & Wachtl (211) have shown that the low glucose oxidation in rabbit lens (accompanying a strong aerobic glycolysis) takes place predominantly via the pentose phosphate cycle. According to Dawes & Holms (212, 213), in *Sarcina lutea* 30 per cent of glucose is decomposed by the pentose phosphate cycle, while 70 per cent is degraded by the glycolytic pathway followed by the citric acid cycle. The preponderant oxidation of glucose via the glycolytic pathway is of particular interest, since this organism is unable to utilize glucose anaerobically.

According to Brin & Yonemoto (214), 85 per cent of the methylene blue-

activated glucose oxidation in erythrocytes occurs via the pentose phosphate cycle. The bottleneck in this reaction sequence is represented by the re-oxidation of TPNH formed on glucose-6-phosphate dehydrogenation. According to King & Cheldelin (215), the enzymes of the pentose phosphate cycle are present in the cytoplasm of *Acetobacter suboxydans*. The particulate fraction from *A. suboxydans* oxidizes nonphosphorylated glucose in a pyridine nucleotide-independent reaction to δ-D-gluconolactone. From the cytoplasm an enzyme has been purified 100-fold which catalyzes the same strictly DPN-specific reaction.

Hochster & Katznelson (216) have demonstrated a glucose-6-phosphate dehydrogenase in *Xanthomonas phaseoli* [just as described in *A. suboxydans* (215) and previously in various other bacteria] utilizing both DPN and TPN. The presence of a pyridine nucleotide transhydrogenase which might simulate a dual specificity was excluded. Though in *X. phaseoli* the enzymes of the pentose phosphate cycle are present, hexose monophosphate is preferentially utilized by the Entner-Doudoroff pathway.

According to Lang & Hartmann (217), phosphoribomutase and phosphoriboisomerase are localized in nuclei. The purification and characterization of ribose-5-phosphate isomerase from spinach leaves and xylulose-5-phosphate epimerase from rabbit muscle are described by Tabachnick *et al.* (208). Dickens & Williamson (218) have shown that formaldehyde, in addition to hitherto described substrates, reacts as an acceptor for the "active glycolaldehyde" produced from hydroxypyruvate by transketolase. The resulting dihydroxyacetone may be phosphorylated with ATP to yield dihydroxyacetone phosphate. Perhaps this reaction may be of physiological importance in connexion with C-1-compound metabolism.

It had been previously shown (219) that erythrose-4-phosphate formed by the pentose phosphate cycle is converted with phosphoenolpyruvate to 5-dehydroquinic acid. Now an aldolase purified by Srinivasan & Sprinson (220) condenses phosphopyruvate with erythrose-4-phosphate to 2-keto-3-deoxy-7-phosphoglucoheptonic acid.

According to Cynkin *et al.* (221, 222) D-ribose, after phosphorylation to ribose-5-phosphate is metabolized in *Clostridium perfringens* by the pentose phosphate pathway. The degradation of L-arabinose by *Aerobacter aerogenes* has been studied by Simpson *et al.* (223, 224, 225). Isomerization to L-ribulose was shown to be the first step (224), and it is followed by phosphorylation with ATP in position 5 catalyzed by an L-ribulokinase purified 200-fold (223). L-Ribulose-5-phosphate is then converted to D-xylulose-5-phosphate by an L-ribulose-5-phosphate-4-epimerase, purified 200-fold (225). In *Lactobacillus plantarum* L-arabinose is metabolized in the same way as in *A. aerogenes* (226, 227, 228). L-Arabinose isomerase was purified by Heath *et al.* (226). The equilibrium mixture contains 90 per cent of L-arabinose and 10 per cent of L-ribulose. Burma & Horecker (227) isolated a kinase which phosphorylates L-ribulose with ATP to L-ribulose-5-phosphate and,

similar to the correponding enzyme from *A. aerogenes* (223), also D-ribulose, though it is less active with the latter substrate. Furthermore, these authors purified an L-ribulose-5-phosphate-4-epimerase catalyzing the reversible conversion with D-xylulose-5-phosphate (228). The equilibrium constant D-xylulose-5-phosphate / L-ribulose-5-phosphate is 1.2 (228) to 1.9 (225).

Hurwitz (229) has reported that in *Leuconostoc mesenteroides* as well as in *L. plantarum* (230, 231) xylulose-5-phosphate produced from glucose by the pentose phosphate pathway is cleaved phosphorolytically to acetyl phosphate and triose phosphate:

$$\text{D-xylulose-5-phosphate} + P_i \rightarrow \text{acetyl phosphate} + \text{D-glyceraldehyde-3-phosphate} \quad (6)$$

Acetyl phosphate may be converted to ethanol, while lactic acid is formed from triose phosphate in the course of fermentation. The enzyme cleaving xylulose-5-phosphate under orthophosphate uptake has been purified from *L. plantarum* by Heath *et al.* (231). The enzyme is designated "phosphoketolase" and requires thiamine pyrophosphate as coenzyme.

Entner-Doudoroff pathway.—The Entner-Doudoroff pathway has been suggested by Hilker & White (232) to be a major pathway in *Endamoeba histolytica*. This organism possesses glucose-6-phosphate dehydrogenase activity, but 6-phosphogluconate dehydrogenase could not be detected. By cleavage of 6-phosphogluconate, phosphoglyceraldehyde and pyruvate are produced. Since triose phosphate dehydrogenase is not present, it is possible that phosphoglyceraldehyde is recombined to form hexose monophosphate, which is again decomposed to pyruvate by the 6-phosphogluconate-splitting enzyme. Stern *et al.* (233) have shown in studies with ^{14}C-labelled glucose, pyruvate, and acetate in five different pseudomonads that probably 70 to 100 per cent of glucose is metabolized by the Entner-Doudoroff pathway.

Glucuronic acid cycle.—Stimulated especially by studies on the origin of congenital pentosuria, research has recently established a new metabolic cycle in animals. This cycle is often called glucuronic acid cycle, according to one of its intermediates (see Fig. 1). Whether only parts of the cycle or whether the whole cycle is of physiological significance has not yet been established. In this connection it must be kept in mind that many of the cross linkages now known to exist among metabolic pathways suggest the formulation of new cycles. However, the decisive question, which is yet to be studied, is whether such a cycle is of quantitative importance.

The first step of the reaction sequence is the conversion of D-glucose to D-glucuronate. A comprehensive presentation of this reaction sequence, in which UDP is involved, was provided by Kalckar & Maxwell (44). Smith *et al.* (234), using chromatographic methods, have shown an enzyme system to exist in pneumococcus oxidizing UDP-glucose to UDP-glucuronic acid. In a particulate fraction of mung bean (*Phaseolus aureus*) seedlings, as well as in shoots, roots, and leaves of other plants, Neufeld *et al.* (235) have
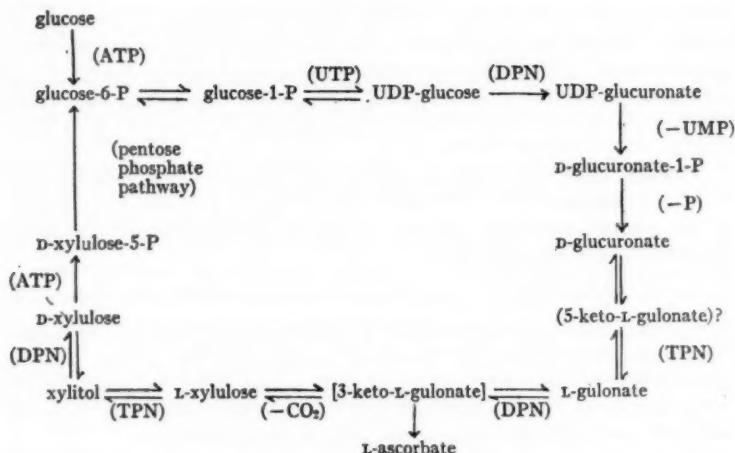
Fig. 1. Glucuronic acid cycle.

demonstrated a 4-epimerase and a decarboxylase converting UDP-glucuron-
ate to UDP-galacturonate and UDP-xylose, respectively. UDP-glucuronic
acid is hydrolyzed to UMP and glucuronate-1-phosphate by a UDP-glucuron-
ate pyrophosphatase, which has been shown to exist in a particulate frac-
tion from rat kidney by Ginsburg *et al.* (236). Glucuronate-1-phosphate is
then hydrolyzed by a phosphatase (236):

(a) UDP-glucuronate + H₂O → UMP + glucuronate-1-phosphate   (7)
(b) glucuronate-1-phosphate + H₂O → glucuronate + phosphate   (8)
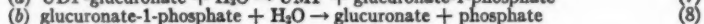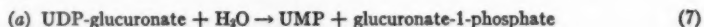
In animals, D-glucuronate can be converted via L-gulonic acid to L-
ascorbic acid. Hassan & Lehninger (103) have shown with an extract from
rat liver that D-glucuronate is reversibly reduced to L-gulonate by means of
TPNH. With rates of the same order, D-galacturonate is reversibly converted
to L-galactonate with preparations from hog kidney (237). The enzyme
from animals is specifically linked to TPN. Kilgore *et al.* (238) purified
an enzyme (uronic acid isomerase) from *Erwinia carotovora* isomerizing
D-galacturonate with 5-keto-L-galactonate (= D-tagaturonate), and sepa-
rated a second enzyme by which 5-keto-L-galactonate is reduced to L-galac-
tonate with either DPNH or TPNH, although the latter is less active:

$$\text{D-galacturonate} \rightleftharpoons \text{5-keto-L-galactonate} \xrightarrow[\text{TPNH}]{\text{DPNH}} \text{L-galactonate} \quad (9)$$
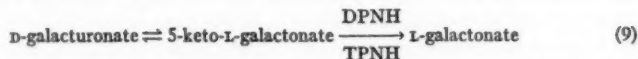
According to Payne & McRorie (239), an analogous sequence of steps was
observed in *Serratia marcescens,* when D-glucuronate is converted to L-

gulonate via the 5-keto compound (5-keto-L-gulonate = D-fructuronate):

$$\text{D-glucuronate} \rightleftharpoons \text{5-keto-L-gulonate} \xrightarrow[\text{TPNH}]{\text{DPNH}} \text{L-gulonate} \qquad (10)$$
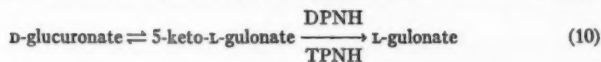
These findings indicate that perhaps in animals D-glucuronate reduction to L-gulonate is preceded by an isomerization (238). According to Ashwell *et al.* (240), further degradation of 5-keto-L-gulonate proceeds in *E. coli* via D-mannic acid to 2-keto-3-deoxygluconic acid, and, analogous to this reaction sequence, 5-keto-L-galactonate is converted to 2-keto-3-deoxygluconic acid via D-altronic acid. In the presence of ATP, 2-keto-3-deoxygluconic acid reacts to yield pyruvic acid and triose phosphate.

L-Gulonate is dehydrogenated with DPN to form the hypothetic intermediate 3-keto-gulonate (237, 241). The latter may be either converted through enolization and lactonization to L-ascorbate, or decarboxylated to L-xylulose. Ashwell *et al.* (241) have continued their studies on the conversion of L-gulonic acid to L-xylulose by enzymes from rat and hog kidney. They purified a DPN-linked L-gulonate dehydrogenase and observed after incubation with L-gulonate a minute accumulation of a ketohexonic acid, presumably identical with 3-keto-L-gulonic acid.

L-Xylulose formed from L-gulonic acid by dehydrogenation and decarboxylation is accumulated in patients suffering from pentosuria, since further conversion to D-xylulose is prevented by a genetic block. That xylulose is formed from glucuronic acid by the same pathway in man as it is in animals was shown by Touster *et al.* (242), who administered ^{13}C-labelled glucuronolactone to a pentosuric human and recovered the isotope from the excreted L-xylulose. Hiatt (243) proved the same in experiments with ^{14}C-labelled glucuronolactone. Touster & Harwell (244) isolated L-arabitol from pentosuric urine. The isotope analysis of this substance after administration of D-glucuronolactone-1-^{13}C is in accord with the fact that L-xylulose, accumulated in pentosuric humans, is partially reduced to L-arabitol.

Normally, L-xylulose is converted via xylitol to D-xylulose by the enzymes described by Hollmann & Touster (245). Then, D-xylulose is phosphorylated to D-xylulose-5-phosphate by a kinase, which was purified 50-fold from liver by Hickman & Ashwell (246). Since the latter compound may be converted to hexose monophosphate by the pentose phosphate cycle, in liver, all the enzymes are present which convert L-xylulose to glucose and glycogen, as was originally proposed by Hollmann & Touster (245). McCormick & Touster (247) have shown this pathway to operate *in vivo*. These authors demonstrated in experiments with guinea pigs and rats that the labelling pattern of glycogen after the administration of xylitol-1-^{14}C is in accordance with the pathway recorded in Fig. 1. Dayton *et al.* (248), after administering [13]C- and ^{14}C-labelled D-glucuronolactone and L-gulonolactone to rats and guinea pigs, have observed, too, a distribution pattern in glycogen accordant with the glucuronic cycle.

OTHER PATHWAYS FOR METABOLISM OF HEXOSES, PENTOSES AND RELATED SUBSTANCES

Glucose.—In *Acetobacter melanogenum* glucose is oxidized via D-glucono-δ-lactone to gluconate without previous phosphorylation and is further oxidized (presumably via 2-ketogluconate) to 2,5-diketogluconate. Datta *et al.* (249), continuing the study of this reaction route, have purified an enzyme from *A. melanogenum* decarboxylating 2,5-diketogluconate. The resulting, extremely sensitive, C_5-compound is converted to α-ketoglutaric acid. From klebsiella and *E. coli* an enzyme had been characterized by de Ley (250) which reversibly reduces 5-ketogluconate to gluconate with both DPNH and TPNH. Possibly in this organism gluconate is first dehydrogenated in the 5-position and thereafter in the 2-position, forming 2,5-diketogluconic acid.

Galactose.—In galactose-adapted yeast, galactose is decomposed by phosphorylation to galactose-1-phosphate and subsequent conversion to glucose-1-phosphate by galactose-1-phosphate transuridylase (also called uridyltransferase) and UDP-galactose-4-epimerase [for summary see (44)]. According to Mills *et al.* (251), of the three enzymes by which galactose is converted to glucose-1-phosphate, only the kinase and the transuridylase are formed in yeast during the adaptation to galactose, while the epimerase could be demonstrated in nonadapted yeast. In contrast to these results, experiments in *Saccharomyces cerivisiae* and *S. fragilis* are presented by De-Robichon-Szulmajster (252, 253) which suggest an adaptive formation of all three enzymes (including the epimerase) in response to galactose.

UDP-glucose pyrophosphorylase from mung bean has been purified approximately 800-fold by Ginsburg (254). The enzyme appears to react specifically with UDP-glucose. According to Neufeld *et al.* (255), in extracts from mung bean seedlings and other higher plants there exist enzymes catalyzing the transfer of the uridyl moiety from UTP not only to glucose-1-phosphate but also to D-galactose-1-phosphate, D-xylose-1-phosphate, and L-arabinose-1-phosphate. In addition, Neufeld *et al.* (255) have demonstrated epimerases which establish an equilibrium between UDP-glucose and UDP-galactose as well as between UDP-xylose and UDP-arabinose. UDP-glucose pyrophosphorylase has been identified by Turner & Turner (256) in extracts from pea seeds, and by Ganguli (257) in homogenates of *Impatiens holstii.*

De Ley & Doudoroff (258) have characterized enzymes from galactose-adapted *Pseudomonas saccharophila* decomposing galactose by a wholly different route, namely by direct oxidation without previous phosphorylation:

$$
\text{D-galactose} \xrightarrow[-2H]{\text{(DPN)}} \text{D-galactono-}\gamma\text{-lactone} \xrightarrow{+H_2O} \text{D-galactonic acid} \xrightarrow{-H_2O}
$$

$$
\text{2-keto-3-deoxy-D-galactonic acid} \xrightarrow{+ATP} \text{pyruvate} + \text{D-glyceraldehyde-3-phosphate} \tag{11}
$$
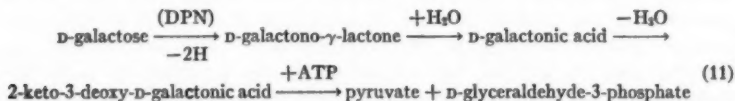
In the last reaction, 2-keto-3-deoxy-6-phosphogalactonate may be formed as an intermediate.

By virtue of tracer experiments with labelled galactose, Bloom (259) has reported that in rat liver the metabolism of this sugar is in accord with earlier described reaction sequences involving galactokinase, galactose-1-phosphate uridyltransferase, and UDP-galactose-4-epimerase. On the other hand, Wood *et al.* (260), investigating the conversion of ^{14}C-labelled glycerol in mammary gland, have found that glucose, as expected, was labelled symmetrically, while galactose exhibited an unsymmetric distribution; this suggests a pathway for galactose metabolism in mammary gland different from that involving glucose. Maxwell (261) has purified UDP-galactose-4-epimerase 200-fold from calf liver. Since catalytic amounts of DPN are required for activity, it appears that the epimerization proceeds via the 4-keto compound. However, attempts to trap the keto compound were unsuccessful. From calf liver, galactose-1-phosphate uridyltransferase has been purified by Kurahashi & Anderson (262). According to Cleland & Kennedy (263), the incorporation of galactose into brain lipides (cerebrosides and sphingomyelin) starts from UDP-galactose. This was demonstrated with homogenates from guinea pig brain.

In patients, suffering from congenital galactosemia, there is a specific deficiency of galactose-1-phosphate uridyltransferase, an enzyme which is involved in the main pathway of galactose metabolism in animals. In spite of this, a small conversion, rising with increase in age, of galactose to glucose is observed. Isselbacher (264) has purified an UDP-galactose pyrophosphorylase [originally described by Kalckar in yeast; cf. also (255)] from beef liver, which will account for this finding. The enzyme catalyzes the following reaction:

$$\text{UTP} + \text{galactose-1-phosphate} \rightleftharpoons \text{UDP-galactose} + \text{pyrophosphate} \qquad (12$$
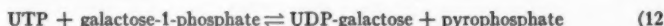
Thus the conversion of galactose to glucose in liver without the participation of galactose-1-phosphate uridyltransferase is rendered possible. The enzyme is distinct from UDP-glucose pyrophosphorylase.

Mannose.—After phosphorylation to mannose-6-phosphate, this sugar enters the pathway of glucose breakdown by isomerization to fructose-6-phosphate. A chromatographic method for separating yeast phosphoglucoisomerase from phosphomannoisomerase is described by Noltmann & Bruns (265). Phosphomannoisomerase from red blood cells has been characterized (266, 267) as an SH-dependent metal-enzyme complex. The enzyme has been demonstrated in a variety of animal tissues.

Fructose.—The pathway of fructose degradation in liver is shown in Fig. 2. In addition to the well-known [for a summary see (268, 269)] reaction sequence leading from D-glyceraldehyde via glycerol and α-glycerol phosphate to the glycolytic pathway, it has been shown (270, 271, 272) that D-glyceraldehyde may be directly dehydrogenated to D-glyceric acid by a

fructose
|
(ATP)
|
fructose-1-phosphate

D-glyceraldehyde ←⟍→ dihydroxyacetone phosphate ⇌ 3-phospho-D-glyceraldehyde

(DPN) (DPN)

(DPN)　glycerol ⟶ α-glycerophosphate
　　　　　　　(ATP)
　　　　　　　　　　(ATP)

D-glycerate ⟶ 3-phospho-D-glycerate

lactate ←⟶ CO₂ + H₂O
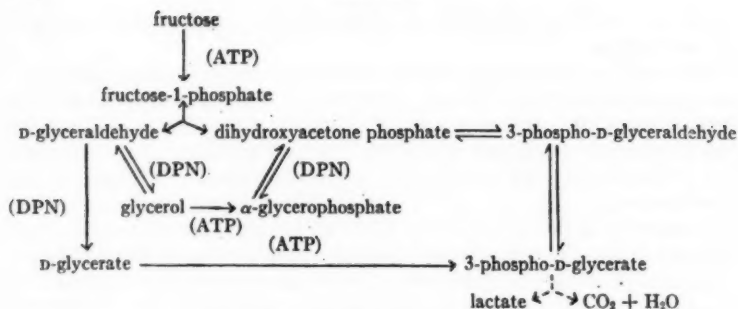
FIG. 2. Fructose degradation in liver.

DPN-specific enzyme. This reaction was observed by Leuthardt *et al.* (270) for the first time. The enzyme in question has now been studied in rat liver by Lamprecht & Heinz (271). The dehydrogenase specifically operates with DPN and shows a high affinity toward glyceraldehyde ($K_M \approx 10^{-4}$ M). Since the relative activity with acetaldehyde and glyceraldehyde does not change during purification, the enzyme dehydrogenating glyceraldehyde is probably identical with the liver acetaldehyde dehydrogenase (271, 272) described by Racker (273) a long time ago. Vanko & Muntz (274) have reported a DPN-dependent D-glyceraldehyde dehydrogenase to be present in rat liver mitochondria. Presumably, this enzyme, too, is identical with Racker's enzyme. Concerning the further degradation of D-glyceric acid, a kinase from rat liver (275) and horse liver (276) has been described by Holzer & Holldorf (275) and by Ichihara & Greenberg (276), phosphorylating D-glyceric acid to D-glyceric acid-phosphate. The latter compound is decomposed further by the classical glycolytic scheme. Glycerol formed by reduction of glyceraldehyde with liver alcohol dehydrogenase (277) is phosphorylated by a glycerokinase crystallized by Wieland *et al.* (278, 279) from pigeon liver. The enzyme may be used to determine glycerol by the spectrophotometric method of Warburg (278).

Fructokinase has now been purified by Parks *et al.* (280) from beef liver. ADP formed in the reaction inhibits fructose phosphorylation. An activation of fructose phosphorylation is observed in the presence of oxygen and intact respiratory chain phosphorylation because of the low level of inhibitory ADP. High ATP concentrations are inhibitory because Mg⁺⁺, which is required for the reaction, is complex-bound. This effect might cause a regulation similar to the Pasteur effect (280, 281). 1-Phosphofruc-taldolase, previously described by Leuthardt *et al.* as occurring in liver, has now been found also in kidney by Wolf & Leuthardt (282). Using ion exchange chromatography, Kaletta-Gmünder *et al.* (283) purified the enzyme from rabbit liver and separated it from fructose-1,6-diphosphate aldolase. Peanasky & Lardy (284) succeeded in crystallizing the enzyme

from bovine liver. It cleaves fructose-1-phosphate at 42 per cent of the rate at which fructose-1,6-diphosphate is cleaved. Dihydroxyacetone phosphate is condensed with formaldehyde at least as fast as fructose diphosphate is cleaved. Contrary to Kaletta-Gmünder et al. (283), Peanasky & Lardy could not obtain any evidence in support of the existence of two different aldolases in liver. The difference of aldolase from liver, kidney, and intestine on the one hand, and from muscle on the other, may be used, according to Wolf et al. (285, 286) and Schapira et al. (287, 288), to identify the origin of serum aldolase in certain diseases.

Williams-Ashman et al. (289) have demonstrated the reversibility of ketose reductase, the existence of which in liver had previously been established, in certain accessory sexual tissues of male rodents. Perhaps, this enzyme, together with a TPN-specific dehydrogenase which reversibly converts sorbitol to glucose, is involved in the formation of fructose from glucose by these tissues, as was described by Hers (cf. 269) for the fructose-secreting seminal vesicle of the sheep.

Fucose and rhamnose.—Ginsburg (290) has shown GDP-mannose to be converted with TPNH to GDP-fucose by extracts of *A. aerogenes*. This reaction, which probably includes several steps, is undoubtedly of importance for the biosynthesis of fucose, since GDP-mannose was detected in yeast (291) and since GDP-fucose was isolated from *A. aerogenes* by Ginsburg & Kirman (292), as well as from milk by Denamur et al. (293). Huang & Miller (294) and Heath (295) have reported that extracts from bacteria isomerize fucose to fuculose and phosphorylate the latter with ATP. Probably, fuculose-1-phosphate is formed, which may be converted to 6-deoxy-L-sorbose-1-phosphate by a phosphoketoepimerase. The latter compound is perhaps cleaved to lactaldehyde and dihydroxyacetone phosphate (294).

Amino sugars.—Pogell & Gryder (296) have characterized an enzyme system from rat liver homogenate which converts D-glucose-6-phosphate with L-glutamine to D-glucosamine-6-phosphate and L-glutamate. Another enzyme, reversibly deaminating glucosamine-6-phosphate to fructose-6-phosphate and NH_3 has been purified 120-fold from *E. coli* and 1360-fold from hog kidney by Comb & Roseman (297). Both deaminating enzymes are activated by N-acetyl-glucosamine-6-phosphate, but isotope and kinetic data of Comb & Roseman suggest that this compound is no intermediate of the reaction, as had been postulated by Leloir & Cardini (298). The equilibrium of the reaction is widely shifted in favor of fructose-6-phosphate and ammonia. A reversal of the reaction, however, may readily be demonstrated if the glucosamine-6-phosphate formed in the reaction is trapped as a result of acetylation by acetyl coenzyme A and a purified acetylase.

Leloir et al. (299) have shown that extracts from rat liver and kidney catalyze the ATP-dependent phosphorylation of acetyl glucosamine to the 6-phosphate, and of acetyl galactosamine to the 1-phosphate.

Muramic acid, the 3-O-lactate ether of acetyl glucosamine, is a con-

stituent of many bacterial cell walls. Strominger (300) has found that extracts from *Staphylococcus aureus* convert UDP-acetyl glucosamine with phosphoenolpyruvate to UDP-acetyl glucosamine pyruvate and orthophosphate. By hydrogenation of the pyruvate compound muramic acid might be produced (300).

By purification of an enzyme from *C. perfringens* decomposing N-acetyl neuraminic acid and by investigation of the reaction products Roseman & Comb (301, 302) have shown that N-acetyl neuraminic acid does not contain the steric configuration of N-acetyl glucosamine but that of N-acetyl mannosamine. The enzyme which was purified 110-fold reversibly cleaves N-acetyl neuraminic acid to yield pyruvate and N-acetyl-D-mannosamine:

$$\text{N-acetyl mannosamine} + \text{pyruvate} \rightleftharpoons \text{N-acetyl neuraminic acid} \qquad (13$$

N-glycolyl neuraminic acid is also cleaved by this enzyme. Concerning the biosynthesis of N-acetyl neuraminic acid, Comb & Roseman (303) present evidence suggesting the existence of the following reaction in rat liver:

$$\text{UDP-N-acetyl glucosamine} \rightarrow \text{N-acetyl mannosamine} \qquad (14)$$
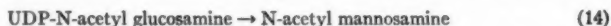
Ethanol oxidation.—In experiments with *E. coli,* De Leon & Creaser (304) could not obtain any evidence that acetaldehyde produced from alcohol during growth is utilized for the synthesis of deoxyribose. Studies by Domagk & Horecker (305), however, suggest that on the breakdown of deoxyribose by adapted *L. plantarum* this sugar, after the phosphorylation to the 5-phosphate, is cleaved to phosphoglyceraldehyde and acetaldehyde by aldolase.

Propionic acid oxidation.—Two pathways are known for the breakdown of propionic acid. The first route leading via β-hydroxy propionate has been studied in peanut mitochondria by Giovanelli & Stumpf (306, 307) and in animal tissues by Kupiecki & Coon (308) and Rendina & Coon (309) as well as by Den (310). Perhaps, the oxidation of propionic acid to β-hydroxy propionic acid proceeds via acrylyl coenzyme A (307, 469), which may be converted by the addition of water and hydrolysis of the thiol ester linkage to β-hydroxypropionic acid (469) and subsequently be dehydrogenated to malonic semialdehyde. The enzyme catalyzing the latter step has been purified 100-fold from pig kidney by Den (310). The reaction is DPN-dependent and reversible. The dehydrogenase was also demonstrated to exist in heart, liver, *E. coli,* etc. Malonic semialdehyde may either be transaminated with glutamate to β-alanine, as was demonstrated by Kupiecki & Coon (308) with an enzyme partially purified from pig kidney, or may be dehydrogenated to malonyl coenzyme A and further decarboxylated to acetyl coenzyme A and CO_2, as was proposed by Giovanelli & Stumpf (307) for peanut mitochondria. In this connection, findings of Walker &

Ladd (311) are of interest, according to which the following conversion of acrylate to propionate has been observed in bacteria from rumen:

$$\text{lactate} + \text{acrylate} \rightarrow \text{pyruvate} + \text{propionate} \qquad (15)$$
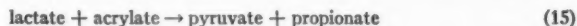
The second pathway of propionic acid oxidation, the enzymes of which were described in detail by Ochoa *et al.* (312, 313, 314) starts with the activation of propionate to propionyl coenzyme A. Involving ATP, propionyl coenzyme A is carboxylated to form methyl malonyl coenzyme A. The enzyme catalyzing this reaction has been purified 200-fold from pig heart by Tietz (315). Methyl malonyl coenzyme A then is converted to succinyl coenzyme A by a transcarboxylating enzyme, described by Beck & Ochoa (316):

$$\text{methyl malonyl coenzyme A} + \text{propionyl coenzyme A} \rightleftharpoons$$
$$\text{propionyl coenzyme A} + \text{succinyl coenzyme A} \quad (16)$$
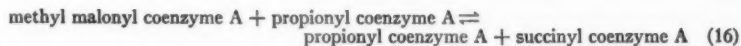
In this reaction the free carboxyl group of methyl malonyl coenzyme A is transferred to propionyl coenzyme A, the latter being converted to succinyl coenzyme A. Thus, the substrate becomes acceptor and the acceptor becomes reaction product, much as in the mechanism of action of phosphate transferring mutases. Tracer experiments have shown that free CO_2 does not participate in transcarboxylation. Succinyl coenzyme A is further decomposed by the citric acid cycle. The finding of Thomas *et al.* (317) that methyl malonic acid occurs in the urine of normal humans stresses the physiological importance of propionic acid degradation via methyl malonic acid.

Hydroxypyruvate.—Interest has been focused on hydroxypyruvic acid particularly in connection with serine metabolism. Lithium hydroxypyruvate is now available in crystalline form (318, 319). A colorimetric method of determining hydroxypyruvic acid has been described by Dickens & Williamson (320), which may also serve to identify glycollic aldehyde. In the spectrophotometric test according to Warburg, Holzer & Holldorf (193), a DPN-specific D-glycerate dehydrogenase from spinach leaves is used to assay hydroxypyruvate without interference from pyruvate. Holzer, Goedde & Schneider (321) have demonstrated that hydroxypyruvate is decarboxylated to glycollic aldehyde by purified pyruvate decarboxylase from yeast. The glycollic aldehyde is reduced to ethylene glycol by DPNH and yeast alcohol dehydrogenase. Erythrulose has been shown to be formed from hydroxypyruvic acid by yeast enzymes, according to Dickens & Williamson (322). The reaction is catalyzed by the co-operation of carboxylase and transketolase (323). By means of phosphorylation and isomerization erythrulose might enter into the pentose phosphate cycle. Apart from hydroxypyruvic acid, glycollic aldehyde is formed from ethanolamine, too. An enzyme catalyzing this reaction has been partially purified by Narrod &

Jakoby (324) from pseudomonads (which oxidize ethanolamine to CO_2, NH_3, and H_2O). Pyridoxal phosphate probably participates in the reaction.

β-Mercaptopyruvic acid is reduced by DPN-linked lactic dehydrogenase from heart muscle (325). Furthermore, an enzyme has been purified from rat liver by Kun & Fanshier (326, 327, 328) catalyzing the desulfuration of β-mercaptopyruvate to pyruvate and sulfur, as well as the transfer of the sulfur of mercaptopyruvate to sulfite forming thiosulfate. The enzyme contains copper; it has been shown to exist in kidney and streptobacillus.

Lactaldehyde.—With the aid of new assay methods for lactaldehyde in the presence of propanediol and acetol it has been suggested by Sandman & Miller (329, 330) that in yeast lactaldehyde can be converted to lactate as well as to propanediol, the latter being reversibly converted to acetol. The oxidation of lactaldehyde to lactate is catalyzed by an aldehyde dehydrogenase, which is, probably, identical with the acetaldehyde dehydrogenase from yeast, known for some time. The reductive conversion of lactaldehyde to propanediol is performed by alcohol dehydrogenase (331).

PHOTOSYNTHESIS

Quantum efficiency, hydrogenation of pyridine nucleotides, and photosynthetic phosphorylation.—Conditions concerning the quantum efficiency in photosynthesis have been summarized by Warburg *et al.* (332). Regularly 3 to 5 moles of quanta are required for the evolution of one mole of oxygen. According to Warburg & Krippahl (333), H_2O cleavage by light (the so-called "Hill reaction") can no longer be considered to be the primary event in photosynthesis. In living and lyophilized chlorella as well as in grana of cabbage turnip leaves, these authors have discovered that for the light-dependent reduction of quinone to hydroquinone, which is accompanied by the evolution of oxygen, the presence of catalytic amounts of CO_2 is indispensable. Warburg & Krippahl (333) conclude that the over-all reaction is therefore to be explained as the sum of the following 2 reactions:

$$2 \text{ quinone} + 2\ H_2O + C \rightarrow 2 \text{ hydroquinone} + CO_2 \qquad (17)$$
$$CO_2 \rightarrow C + O_2 \qquad (18)$$

$$\text{sum: } 2 \text{ quinone} + 2\ H_2O \rightarrow 2 \text{ hydroquinone} + O_2 \qquad (19)$$
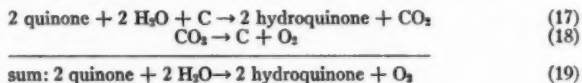
In this reaction sequence, quinone substitutes molecular oxygen which in normal photosynthesis is required for the oxidation of part of C (C signifies reduction products of CO_2) to supply the energy, necessary in addition to light energy, for CO_2 cleavage. Already Brown & Frenkel (334) have shown in a review that the tracer oxygen experiments often cited to prove the origin of photosynthetic O_2 from H_2O do not furnish conclusive evidence.

The view prevailing at present holds that light has to supply ATP and reduced coenzymes to render possible the photosynthetic fixation of CO_2. ATP and reduced coenzymes are required to reduce CO_2 to carbohydrates in dark reactions. Previously it was assumed that ATP production in photo-

synthesis is performed by the mitochondrial oxidation of reduced substrates by means of respiratory chain phosphorylation, which is well known to occur in animal tissues. However, the experiments of Arnon *et al.* [for more recent summaries see (335, 336)] have afforded evidence that in the chloroplasts of green plants a "photosynthetic phosphorylation" takes place which—though very similar to respiratory chain phosphorylation—is essentially distinct from it. Vitamin K, flavin mononucleotide, and ascorbate are required for photosynthetic phosphorylation, which was investigated in the particles of some microorganisms and a variety of plants (337 to 340). Phenazine methosulfate is frequently used as an artificial oxidant (337, 341); its high activity in photosynthetic phosphorylation (341) was found by Walker & Hill (342) to result from its conversion to pyocyanine. In more recent papers, Arnon *et al.* (335, 336) have demonstrated that TPN, too, is required as a cofactor for photosynthetic phosphorylation. According to Trebst and co-workers (335), the production of one mole of ATP from ADP and phosphate is stoichiometrically related to the reduction of one mole of TPN. On the reoxidation of reduced TPN with the "oxidizing agent," involving flavin, vitamin K, ascorbic acid, and cytochrome enzymes, perhaps, more ATP is synthesized. The reduction in the light of pyridine nucleotides, which had already previously been observed, has been confirmed and further investigated by many authors (343 to 348) with algae, grana from higher plants, and chromatophors from rhodospirillum.

According to Frenkel (349), a light-dependent stoichiometric reduction of DPN at the cost of reduced flavin mononucleotide has been observed to be catalyzed by chlorophyll-containing chromatophors from *Rhodospirillum rubrum*. Perhaps in this process the reversal of respiratory chain phosphorylation [proposed some time ago (350)] is involved: ATP is generated in the light by means of photosynthetic phosphorylation, and with the aid of this ATP hydrogen is pumped to a more negative potential.

It is taken for granted at present that in green plants the energy of light can be used to form TPNH and ATP. Whether ATP production exclusively takes place in the chloroplasts in the course of photosynthetic phosphorylation (351) or whether normal dark respiration by means of mitochondrial respiratory chain phosphorylation is also involved cannot as yet be stated with certainty. More recent experiments of Warburg & Krippahl (352) favor a participation of dark respiration. These authors found perfectly congruent curves for the dependence of photosynthesis, on the one hand, and dark respiration, on the other, on the oxygen tension.

The path of carbon.—The operation of the "reductive pentose phosphate cycle" (353) as a pathway of CO_2 incorporation in photosynthesis is suggested by many experiments. Bergmann *et al.* (354) presume that CO_2 fixation in chemoautotrophic bacteria also proceeds predominantly by this pathway via phosphoglyceric acid to hexose monophosphate. The key enzyme of the reductive pentose phosphate cycle is the enzyme, called carboxydismutase or ribulose diphosphate carboxylase, converting ribulose diphosphate

with CO_2 to two molecules of phosphoglycerate. Moses & Calvin (355) have obtained evidence from paper chromatographic investigations that the first reaction product of this carboxylation is represented by 2-carboxy-3-ketopentitol-1,5-diphosphate. After illumination of algae in the presence of radioactive CO_2, a radioactive spot has been found which must probably be attributed to this substance. In addition, the corresponding 4-keto compound is formed which in the view of these workers is likely to be an artifact.

Metzner et al. (356, 357) have reported that new labile fixation products are obtained when illuminated algae suspensions are fixed with cold acetone. Kandler (358) has communicated experiments according to which the killing of algae, usually performed with hot alcohol, probably simulates a too high fixation of $^{14}CO_2$ in phosphoglyceric acid.

Experiments by Gibbs & Kandler (59) indicate that the distribution of ^{14}C activity in the hexose produced photosynthetically from $^{14}CO_2$ is not in accordance with a symmetric synthesis from two molecules of triose phosphate. The activity of the carbon 4 of glucose derived from starch and sucrose is more pronounced than the activity of carbon 3. Since under anaerobic conditions, formic acid is preferentially incorporated at carbon 4 of the hexose, it was recently supposed by Gibbs (359) that photosynthesis leads from carbon dioxide via formic acid to the formaldehyde level, the latter being transferred to a C_5- or C_2-acceptor to form hexose or triose. Also Mortimer (360) concludes from observations on the cyanide inhibition of photosynthesis that some mechanism other than the reduction of phosphoglyceraldehyde to phosphoglyceric acid is involved in the pathway from CO_2 to hexoses.

Undoubtedly, in green plants several reactions of CO_2 fixation are proceeding simultaneously. Interest was focused by Warburg et al. (361, 362), as well as by Vishniac & Fuller (363), on the carboxylation of γ-aminobutyric acid to glutamic acid. Rosenberg et al. (169) have demonstrated that in spinach chloroplasts CO_2 fixation to phosphoenolpyruvic acid is very active. In this reaction aspartic acid is formed via oxaloacetic acid by transamination.

ENZYMATIC REGULATION

Recent reviews have been published by Krebs & Kornberg (364), Krebs (365), and Holzer (366). In the summer of 1958 a symposium on this topic was held by the Ciba Foundation (367). By enzymatic regulation ("regulation on the level of enzymes") are understood regulative mechanisms which determine, by means of the co-operation of enzymes in intact cells, the rate of metabolism and which are responsible for the changes in the rate of metabolism which appear when conditions are altered. These changes of metabolism may consist of accelerations or retardations, as well as of shifts of metabolism from one pathway to another. Such regulative mechanisms on the level of enzymes ought to be distinguished from "hormonal" and

"nervous" regulations. Nonetheless, many mechanisms of hormonal and nervous regulation may eventually be shown to rest on enzymatic regulation. In principal, enzymatic regulations may be brought about by changes in enzyme activity: by inhibition or activation of an enzyme as well as by increase or decrease in enzyme concentration. However, the more interesting changes in metabolism, caused by enzymatic regulation, are those in which the activities of the involved enzymes remain unchanged. Some prerequisites for regulations of this kind are: (*a*) the presence of feedback mechanisms in a reaction chain; (*b*) the competition of several enzymes for the same substrate or coenzyme, as when branching points of metabolism are present and when a transferring coenzyme participates in different enzyme reactions; (*c*) the "compartmentation" of enzymes, coenzymes, and metabolites.

General studies on the co-operation of the enzymes of carbohydrate metabolism.—For quantitative kinetic statements, e.g., for the calculation of the distribution of the substrate at a branching point of reaction chains, it is necessary to know the *in vivo* concentrations of the metabolites involved. This requirement continues to present great difficulty, since in no case is the size of the "dissolving space," in which the metabolites are contained within the cell, known exactly.

An analysis of the equilibrium $K =$ glucose-1-phosphate/orthophosphate catalyzed by phosphorylase had previously been accomplished by Trevelyan *et al.* (368) in yeast and was repeated by Lynen (369, 370) to calculate the "true" concentration of orthophosphate. Since the orthophosphate concentration determined by conventional methods is markedly higher than the value calculated from the analysis of glucose-1-phosphate and from the equilibrium of phosphorylase, the authors suppose the orthophosphate to be distributed within the cell very disproportionately.

By the use of indirect methods, data can be obtained on the quotient of the concentrations of DPNH and DPN in living cells (371). In yeast cells, DPN and DPNH are in equilibrium with alcohol and acetaldehyde catalyzed by alcohol dehydrogenase, which is present with high activity. Provided the equilibrium constants and the pH are known, the quotient DPNH/DPN can be calculated from the analysis of the concentrations of alcohol and acetaldehyde. Amazingly, quotients of 1:1000 for DPNH/DPN resulted, while previous determinations by various workers based on spectrophotometric analysis (376) or assays after destroying the cells by heat, acid, or alkali demonstrated values of 1:2 to 1:5. These discrepancies must be attributed to the fact that by the indirect method (analysis of the alcohol dehydrogenase equilibrium) the concentrations of free and diffusible, i.e., thermodynamically active, DPNH and DPN are estimated, whereas the direct methods comprehend the sum of free and protein-bound nucleotides. Experiments by Duysens & Kronenberg (372), based on the estimation of the fluorescence spectrum of both free and alcohol de-

hydrogenase-bound DPNH, show that in living yeast cells actually a very appreciable fraction of DPNH is bound to protein and is not present as free DPNH.

Even though the true metabolite concentrations in intact cells are not known, often very interesting statements on the co-operation of enzymes can be made on the basis of changes in concentrations. Thus, Syrett (373) has measured the changes in ATP concentration after the addition of glucose to starved cultures of chlorella. A characteristic traverse of a minimum before a new steady state concentration is reached has been observed. This was noted, too, by Holzer (374) and Holzer & Freytag-Hilf (375) after the addition of glucose to starved yeast cells. Such a traverse of concentrations of intermediates through a minimum or a maximum caused by a change of conditions has been described, e.g., by Chance (376) for the behavior of DPNH and by Holzer (344, 366, 375) for fructose diphosphate, triose phosphate, pyruvate, ATP, and ADP after glucose addition to starved yeast. Without doubt, the appearance of such a maximum or minimum indicates that the quotient

$$Q = \frac{\text{supply of the metabolite investigated}}{\text{consumption of the metabolite investigated}}$$

changes from a value greater than one to less than one and vice versa. Experiments of this kind show that the rate-limiting function of a reaction sequence may be transferred from one enzyme to another. This has to be borne in mind, if one goes in search of the "pacemakers" (364, 365) in a reaction sequence.

Distribution of carbohydrate metabolism to different pathways.—The extent of carbohydrate metabolism and the distribution to different pathways depends primarily upon the "enzyme spectrum," i.e., upon the activities of the different enzymes in a certain type of cells. Therefore, investigations on the distribution of enzymes in different types of cells are of outstanding interest. In interpreting the results of such experiments, however, it should be kept in mind that some enzymes are present in excess, so that changes in activity within far limits do not influence the over-all rate, while any activity changes of the rate-limiting enzymes ("pacemakers") will immediately bear on the over-all rate of the reaction sequence.

The isotope technique, using glucose labelled at various C atoms, presents valuable data concerning the problem of the distribution of carbohydrate metabolism to different pathways. By this method Wang *et al.* (377) have shown that in *S. griseus* the oxidative degradation of glucose takes place predominantly by means of the glycolytic pathway followed by the citric acid cycle, while only a very small amount is oxidized via pentose phosphate pathway. Possibly the age of the cells influences this distribution of glucose catabolism. According to Beck (378), in leucocytes, too, less than 10 per cent of oxidative glucose degradation proceeds by the pentose phosphate cycle. In leukemic cells the share of the pentose phosphate path-

way was found to be slightly higher. Beck could demonstrate that this may probably be attributed to a lower hexokinase activity in leukemic cells; this results in a lower steady state concentration of glucose-6-phosphate and thus favors the degradation via the pentose phosphate cycle before the degradation via glycolytic pathway, because of the different Michaelis constants of glucose-6-phosphate dehydrogenase and phosphofructokinase. By the same author the activities of the dehydrogenases of both the pentose phosphate cycle and the glycolytic pathway in the presence of excess substrate were found to be 10 times higher than is required for their metabolic function in the intact cell (402). This observation underlines Krebs & Kornberg's (364) statement that in most cases of biological oxidation the enzyme activities are not rate controlling.

In hepatoma as well as in normal, embryonic, and regenerating liver, Weber & Cantero (379) have determined the activities of the four enzymes utilizing glucose-6-phosphate (glucose-6-phosphatase, phosphoglucomutase, phosphohexoisomerase, and glucose-6-phosphate dehydrogenase), thus channelling glucose degradation into different pathways. In hepatoma the activities of glucose-6-phosphatase and phosphoglucomutase are greatly decreased, while glucose-6-phosphate dehydrogenase activity is extremely increased. In drawing conclusions from such determinations of enzyme activities with respect to the distribution of glucose metabolism to different pathways, it has to be considered, however, that at low glucose-6-phosphate concentrations the different Michaelis constants of the enzymes cause a distribution pattern different from that obtained on saturation with glucose-6-phosphate. Moreover, in all types of cells hitherto investigated the activity of the enzyme phosphohexoisomerase was shown to be so high that glucose-6-phosphate and fructose-6-phosphate are always found to be at equilibrium. Therefore, this enzyme cannot be said to have a rate-controlling function in metabolism.

Allen & Powelson (380) have studied whether the shift toward the pentose phosphate pathway, characteristic of the onset of growth, is associated with the occurrence of cell division. Cell divisions in cultures of *E. coli* were selectively inhibited by 5-diazouracil without influencing the rate of growth. The authors did not observe any shift of glucose breakdown from the pentose phosphate cycle toward the glycolytic path. This suggests that the pentose phosphate cycle is characteristic of growth, independent of the presence of cell divisions.

Regulation of glycolysis and respiration.—At present, the explanation for the inhibition of glucose utilization by oxygen (Pasteur effect) is generally based on the theory proposed by Lynen (381) and Johnson (382). According to these authors, a lack of orthophosphate and ADP under aerobic conditions resulting from the intensive respiratory chain phosphorylation is responsible for the inhibition of triose phosphate dehydrogenation. Still unclarified, however, remains the question why under aerobic conditions the phosphorylation of glucose with ATP by the hexokinase reaction is re-

pressed, though the Lynen-Johnson theory implies a high ATP level. Experiments by Lynen (369, 370) and by Holzer, Witt & Freytag-Hilf (383) on the change in the concentrations of orthophosphate, ADP, ATP, glucose-6-phosphate, fructose-1,6-diphosphate, pyruvate, and acetaldehyde on transition from anaerobic to aerobic conditions and vice versa support the theory established by Lynen & Königsberger in 1951 (384) that ATP accumulates aerobically in the mitochondria and thus is lacking in the cytoplasm, where it is required for the phosphorylation of glucose by means of hexokinase. In addition to this possibility, which rests on a segregation of the enzymes involved in glycolysis and oxidative phosphorylation, the formation of a complex of ATP with Mg^{++} may be considered. This might be responsible for the fact that, in spite of high ATP concentrations, the phosphorylation of glucose is depressed (269, 281, 385, 386).

Investigations by Racker (387) and Kvamme (388) with Ehrlich ascites tumor cells are consistent with the theory of Lynen and Johnson that the concentration of inorganic phosphate is a limiting factor of anaerobic and aerobic glycolysis. Also Balazs & Richter (389), working with brain homogenates, present evidence for a rate-limiting role of triose phosphate dehydrogenase. Korff & Twedt (390) have demonstrated that the oxidation of phosphoenolpyruvate by mitochondria (with added pyruvate kinase) may be inhibited by the lack of ADP resulting from respiratory chain phosphorylation. Hence, a lack of ADP might play a role limiting the rate of degradation of glucose, not only in the phosphoglycerate kinase reaction, but also in the pyruvate kinase reaction.

According to van Potter & Niemeyer (391), an acceleration of the direct oxidation of hexose monophosphate may lead to an inhibition of glucose breakdown by the glycolytic pathway, since 6-phosphogluconic acid, formed by dehydrogenation of glucose-6-phosphate, inhibits the isomerization of glucose-6-phosphate to fructose-6-phosphate. Moreover, the glucose-6-phosphate accumulated as a result of the inhibition of hexosemonophosphate isomerase might inhibit the hexokinase reaction in tissues in which a glucose-6-phosphate-sensitive hexokinase is present. Whether this mechanism is of importance *in vivo* has yet to be elucidated.

The observation, often called Crabtree effect (392), that on incubation with glucose tumor tissues show a decreased respiration is attributed by Chance *et al.* (393, 394) to an inhibition of respiration caused by a lack of phosphate and ADP. Medes & Weinhouse (395) confirm the finding of Slechta *et al.* (396) that mainly an oxidation of fat is involved in the endogenous respiration of ascites tumor cells. These authors suggest that the Crabtree effect is caused by a depression of fat oxidation in the presence of glucose. Of interest in this connection is the observation of Miroff & Cornatzer (397) that the decrease in respiration of ascites tumor cells in Krebs-Ringer solution caused by larger concentrations of glucose may be prevented by the addition of serum proteins. Possibly these results are related to the observation by Warburg *et al.* (398) that various animal tissues

suspended in homologous serum do not exhibit any aerobic glycolysis, whereas a strong aerobic glycolysis appears in saline solutions. If embryonic tissue, which normally does not show aerobic glycolysis, is grown in tissue cultures, then aerobic glycolysis appears as an artifact produced by unphysiological conditions (399, 400).

High rates of aerobic glycolysis were measured both in normal human leucocytes and in leucocytes of myelocytic and lymphocytic leukemia (401, 402). These measurements were not carried out in serum, but in saline solutions as a suspending medium. In manometric investigations of leucocytes, which were prepared very carefully and suspended in homologous serum, Warburg *et al.* (403) could not detect any aerobic glycolysis at all. Beck (402) has studied intensively the glycolytic rates in homogenates of different types of leucocytes and has found the hexokinase activity to be decisive for the rate of glycolysis. Neufach & Melnikowa (404) have shown hexokinase and phosphofructokinase to be the least active rate-limiting enzymes of glycolysis in muscle extracts.

In the view of a variety of authors a therapeutic effect on tumors may be gained by an inhibition of glycolysis. Warburg *et al.* (405, 406) have shown that x-rays cause an inhibition of glycolysis, and that this inhibition can be accounted for quantitatively by the action of hydrogen peroxide, which is produced by irradiation. According to Holzer & Frank (407), the inhibition of glycolysis by hydrogen peroxide is caused by a decrease of DPN concentration. Nicotinamide prevents the inhibiting action of hydrogen peroxide by maintaining a high DPN level. Carcinostatically active ethylene imine compounds (408 to 412) diminish the DPN concentration in ascites tumor cells and in solid tumors and thereby cause an inhibition of glycolysis which might be responsible for the carcinostatic effect. A depression of glycolysis has also been observed in the action of carcinostatic quinone and quinoline derivatives (413) as well as nitrogen mustard compounds (414) on ascites tumor cells. Morton (415) believes that the DPN-pyrophos-phorylase-catalyzed synthesis of DPN which takes place predominantly in the cell nucleus is decisively involved in the regulation of growth. The possibility of influencing malignant growth by influencing the rate of DPN synthesis is suggested by this author. Burk *et al.* (416, 417) suggest the chemotherapeutic effect of some carcinostatica to be brought about by inhibition of glucose phosphorylation.

The intercorrelations between aerobic fermentation and the biochemical processes of growth in yeast cells have been studied in detail by Holzer and his co-workers (374, 412, 418). If in glucose-oxidizing yeast cells, growth is initiated by the addition of ammonium ions, then the aerobic fermentation is very rapidly enhanced. The increase is probably caused by the following mechanism: the added ammonium is fixed by α-ketoglutarate to form glutamate. From glutamate other amino acids are formed by transamination, and these make possible the ATP-consuming synthesis of peptides and proteins. Because of the consumption of ATP, orthophosphate and ADP are

liberated, and thus, according to the theory of Lynen and Johnson, a stronger fermentation may take place. The alterations of the metabolite concentrations in question could be demonstrated in yeast cells to be correspondent to this idea. Schmid (419) has shown that in baker's yeast there may exist conditions under which growth and cell multiplication occur without aerobic fermentation. By studying the metabolites necessary for growth, after blocking triose phosphate dehydrogenation with iodoacetic acid, Schmid has found that the operation of the citric acid cycle and the formation of pentose phosphate either by the oxidative pathway or anaerobically by the transketolase-transaldolase reaction are indispensable contributions of carbohydrate metabolism to cell multiplication. Experiments of Holzer & Witt (420) have likewise demonstrated that the initiation of growth by the addition of NH_4^+ ions to glucose-oxidizing yeast cells is followed by a very strong acceleration of direct glucose oxidation via the pentose phosphate pathway. Thus, growth is promoted by the supply of pentose phosphate. This self-regulation of carbohydrate metabolism is brought about by the following mechanism: due to the addition of NH_4^+ ions, α-ketoglutarate is reductively aminated by a TPN-specific glutamate dehydrogenase occurring with high activity in yeast, thus furnishing oxidized TPN, which renders possible an intensified dehydrogenation of glucose-6-phosphate.

TPNH reoxidation as a rate-limiting step of pentose phosphate cycle.— Several authors have shown that TPN is present preferentially in its reduced form in all cells and tissues hitherto studied (62, 114, 421). This has to be attributed to the fact that the sum of the processes utilizing TPNH (such as the hydrogenation of crotonyl coenzyme A, mevalonic acid, and glutathione; the reductive amination of α-ketoglutarate; the reductive fixation of carbon dioxide to pyruvate yielding malate; and the hydroxylation of phenylalanine to tyrosine) is less active than the two TPNH supplying dehydrogenating steps of the oxidative pentose phosphate pathway. Thus, it becomes comprehensible that the operation of the pentose phosphate cycle, which requires the presence of oxidized TPN, is controlled by all processes oxidizing TPNH. Kinoshita (422) has found evidence for the operation of this regulative mechanism in experiments with bovine corneal epithelium, in which pyruvate was added as an acceptor for the hydrogen of TPNH. Studies by Hers (269, 423) with rabbit liver slices to which TPNH-reducible glucosone, glyceraldehyde, or glucuronolactone respectively, had been added, led to the same results. As a consequence to the initiation of the reoxidation of TPNH, in the experiments of Hers and Kinoshita, an acceleration of the pentose phosphate cycle could be demonstrated by an increased liberation of $^{14}CO_2$ from glucose-1-^{14}C. In analogous studies Cahill *et al.* (424) have shown that the addition of methylene blue or pyocyanin to rat liver slices stimulated the oxidation of glucose via the pentose phosphate pathway by the reoxidation of TPNH. Also in glucose-oxidizing yeast cells the operation of the pentose phosphate pathway is accelerated by processes reoxidizing TPNH, as was mentioned above (420).

A further possibility for the oxidation of TPNH may consist in a hydrogen transfer to DPN catalyzed by pyridine nucleotide transhydrogenase. Since most DPN, in contrast to TPN, occurs in its oxidized form in all cells and tissues investigated as yet, there exists an energetic potential favoring the hydrogenation of DPN at the cost of TPNH. On the basis of determinations of lactic dehydrogenase activity with TPNH and DPNH respectively, Navazio et al. (425) and Kinoshita (422) have considered a hydrogenation of pyruvate with TPNH as the cause for a stimulation of TPN-specific dehydrogenation reactions and as a mediate in the oxidation of TPNH by mitochondria. Holzer & Schneider (426) have demonstrated that crystalline lactic dehydrogenase from rabbit muscle catalyzes the hydrogen transfer from TPNH to DPN according to the following equations:

$$TPNH + H^+ + pyruvate \rightleftharpoons lactate + TPN^+ \tag{20}$$
$$DPN^+ + lactate \rightleftharpoons pyruvate + DPNH + H^+ \tag{21}$$
$$\text{sum: } TPNH + DPN^+ \rightleftharpoons TPN^+ + DPNH \tag{22}$$
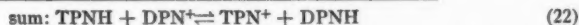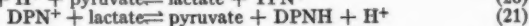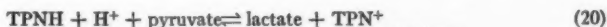
With crystalline glutamate dehydrogenase from ox liver the hydrogen transfer from TPNH to DPN could be demonstrated too (426), since the enzyme functions with DPN as well as with TPN. Another possibility of transferring hydrogen from TPNH to DPN has been shown by Talalay et al. (427, 428). Hydroxysteroid dehydrogenase from human placenta, which has dual nucleotide specificity, catalyzes the transfer of hydrogen from TPNH to DPN and vice versa at a concentration of 10^{-8} M estradiol-17β, the latter being reversibly converted to estrone in this reaction. A hydrogen exchange between TPN and DPN is also catalyzed by 3α-hydroxysteroid dehydrogenase (429), in addition to the 17β-hydroxysteroid dehydrogenase just mentioned. It is possible that the extremely fast dephosphorylation of TPNH to DPNH observed in homogenates of rodent prostate gland and seminal vesicle is also involved in hydrogen transfer (430).

DPNH reoxidation as a rate-limiting step.—Since DPN in most living cells occurs mainly in its oxidized form, the oxidation of DPNH is unlikely to play a rate-limiting role. During a short initial phase, however, in the anaerobic fermentation of yeast cells, DPNH has been found to accumulate (374, 375). In this phase a lack of oxidized DPN limits the degradation of glucose. This must be ascribed to the fact that during the first minutes of fermentation there is no acetaldehyde available to accept the hydrogen from DPNH, and only when a sufficient amount of acetaldehyde has accumulated can DPNH be reoxidized at the rate required for steady state fermentation. Spectrophotometric findings of Chance (376) are perfectly consistent with these observations: during the first minutes of yeast fermentation, reduced DPN is accumulated; then it decreases to a lower steady state concentration.

Investigations by Bücher et al. (97, 431, 432) have furnished evidence that the oxidation of DPNH with oxygen by the mitochondria of insect flight muscles cannot take place directly but must be mediated by α-glycero-

phosphate dehydrogenase localized in cytoplasm and by α-glycerophosphate oxidase present in mitochondria, co-operating according to the following equations:

Cytoplasm: DPNH + dihydroxyacetone phosphate $\rightleftharpoons$ α-glycerophosphate + DPN   (23)

Mitochondria: α-glycerophosphate + $\frac{1}{2}$ $O_2 \rightarrow$ dihydroxyacetone phosphate + H_2O (24)

$$\text{sum: DPNH} + \tfrac{1}{2} O_2 \rightarrow \text{DPN} + H_2O \qquad (25)$$
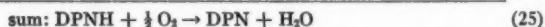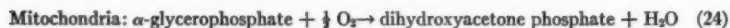
According to this, the hydrogen of DPNH is transported from the cytoplasm to the mitochondria with α-glycerophosphate as a carrier.

Miscellaneous—A possibly rate-limiting role of substrate penetration into cells can be reviewed only in brief. In this context Cohen & Monod (124) assume the operation of enzymelike transport mechanisms ("permeases"). Sols *et al.* (433) described experiments in which a "hexose transportase" is thought to be rate-limiting in anaerobic yeast fermentation. Lynen (369, 370), however, has shown for yeast cells that the rate of glucose penetration is independent of aerobic or anaerobic conditions. Therefore, an aerobically and anaerobically different rate of transport of glucose across cell membranes cannot explain the Pasteur effect.

There is room for only a few remarks on adaptive synthesis of enzymes as a mechanism to control the rate and the pathways of carbohydrate metabolism. Experiments of Freedland & Harper (434, 435, 436) have shown that the glucose-6-phosphatase activity of rat liver is increased in response to a decreased glucose intake. Since this change of activity can be prevented by ethionine, probably a *de novo* synthesis of this enzyme takes place, rather than the activation of a precursor. Similarly, the experiments of Landau *et al.* (437) are felt to be consistent with an increase in glucose-6-phosphatase in galactose (instead of glucose) fed rats. In this way, glucose-6-phosphatase accommodates itself to the amount of glucose supplied by food, thus facilitating the maintenance of a constant level of blood sugar.

Harary (438) has demonstrated that in muscle, yeast, and liver 1,3-diphosphoglyceric acid is hydrolyzed to 3-phosphoglyceric acid. The enzyme, purified 600-fold from muscle, also catalyzes the hydrolysis of other acyl phosphates. Since the acyl phosphatase uncouples ATP synthesis from triosephosphate dehydrogenation, a regulation of the rate of glycolysis by this enzyme might be taken into consideration. More recent experiments by Harary (439) indicate the possibility that acyl phosphatase is involved in the action of the thyroid hormones. In hyperthyroid rats both muscle and liver showed an increase of about 40 per cent in acyl phosphatase activity.

HORMONAL REGULATIONS

Insulin.—Levin & Weinhouse (440) studied the very marked acceleration of incorporation of radioactivity from ^{14}C-labelled glucose into skeletal muscle glycogen caused by the administration of insulin. In contrast, no effect was seen as to the incorporation of labelled glucose into liver glycogen.

According to Spiro *et al.* (441) it is not justified to draw from such experiments the conclusion that insulin does not primarily affect liver. These authors used alloxan-diabetic animals, all metabolic functions of which were restored to normal after two weeks of insulin treatment, thus representing a hypoinsulin state without other damages (442). If such animals adapted to exogenous insulin are acutely deprived of insulin, the alterations of carbohydrate metabolism characteristic of diabetes appear in the same chronological sequence in diaphragm as well as in liver (441). Shaw & Stadie (443), in previous experiments with diaphragm, have shown insulin to accelerate glycogen synthesis from glucose, while the formation of lactate from glucose does not respond to insulin. Further recent experiments by these authors (444) have demonstrated that lactate production, too, is stimulated by insulin after bicarbonate treatment of the diaphragm. Shaw & Stadie (443) deduce from their experiments the coexistence of two glycolytic systems in diaphragm which differ in their response to insulin. Winegrad & Renold (445) report that insulin enhances glucose uptake, glucose oxidation to CO_2, and lipogenesis of adipose tissue *in vitro*. These effects were apparent within 15 min. after incubation with insulin. These authors, too, have noted that an insulin-stimulated lactate formation from glucose is only obtained in bicarbonate buffer as the suspending medium. Measurements of the P/O ratio in hepatic mitochondria from the diabetic cat by Vester & Stadie (446) indicate a significantly decreased P/O ratio in diabetes. Whether this is a secondary effect, or whether insulin is required for the perfect operation of the respiratory chain has yet to be clarified.

The ketone substances formation in diabetic liver is often said to be caused by a lack of oxaloacetate resulting from a decreased glucose breakdown. According to this theory, acetyl coenzyme A is accumulated, since there is no oxaloacetate available for the condensation to citric acid. The accumulated acetyl coenzyme A causes an increase of acetoacetyl coenzyme A, followed by the formation of free acetoacetic acid. The latter reaction proceeds after Lynen *et al.* (447) via β-hydroxy-β-methylglutaryl coenzyme A. However, oxaloacetate determinations by Tapley & Kalnitsky (448, 449), employing a particularly sensitive assay method, contradict the theory that a diminished oxaloacetate concentration in liver is a cause for ketosis. These authors found that in rats fasted for 24 hr. the oxaloacetate concentration in liver is as high as in controls, though the formation of acetoacetic acid has increased 10-fold. Therefore, at least in the case of starvation diabetes, a significant ketosis without change in the concentration of oxaloacetate is possible. According to Lynen (467) and Wieland (468), the energetic situation controlling metabolism via the adenylic acid system has a share in the occurrence of diabetic ketosis. The liver has to produce 1.6 times as much acetyl coenzyme A from fat as from carbohydrate to obtain the same amount of ATP (7 equivalents of ATP are produced between carbohydrate and acetyl coenzyme A and only 4.3 equivalents of ATP are formed between fat and acetyl coenzyme A). Therefore, because of the impaired carbohydrate

metabolism, the diabetic organism is flooded with acetyl coenzyme A; since it can be only partly burned by the citric acid cycle, there is accumulation of coenzyme A and then formation of ketone substances.

A direct attack of insulin on the phosphorylation of glucose has not yet been demonstrated *in vitro* with the necessary accuracy and reproducibility. More recent experiments by Kipnis (450) with 2-deoxyglucose, which is phosphorylated to the 6-phosphate in diaphragm and accumulated as such, indicate that insulin stimulates both the transport and the phosphorylation of glucose. According to these experiments, epinephrine, in contrast to insulin, activates only the phosphorylation. Riklis & Quastel (451, 452) found that the transport of glucose across guinea pig intestine, when small concentrations of glucose are used, is activated by potassium ions to a rate which otherwise is attained only with high concentrations of glucose. This activation is inhibited by dinitrophenol. A transport of glucose linked to a phosphorylation, which is somehow potassium-dependent, might account for these experiments.

Other hormones.—The action of glucagon and epinephrine on the formation of phosphorylase from dephosphophosphorylase previously demonstrated in liver homogenates may be used, according to Berthet *et al.* (453), to perform determinations of purified glucagon preparations. Davidson & Salter (454) have shown that the oxygen consumption of rats is stimulated by glucagon. This effect cannot be explained by a secondary increase of the blood glucose level following glucagon administration, since a hyperglycemia resulting from glucose injection causes only a minute stimulation of oxygen uptake. Perhaps the adrenal glands are involved in this metabolic response to glucagon, since adrenalectomized rats do not show any effect.

Various recent findings support the assumption that thyroxin acts by uncoupling the respiratory chain phosphorylation. In this connection, an acceleration of glucose breakdown via the pentose phosphate pathway as well as via the glycolytic pathway could be demonstrated by Spiro & Ball (455) in hyperthyroid rats. According to Gutenstein & Marx (456), the glucose oxidation in yeast as well as in *A. aerogenes* can be increased by L-thyroxin addition. Thyroxin analogues inhibit, according to Cereijo-Santalo *et al.* (457), the respiration of ascites tumor cells and thus increase aerobic glycolysis. Whether the finding of Wolff & Wolff (458) that pig heart malic dehydrogenase and other dehydrogenases are inhibited by thyroxin is of importance for the mechanism of action of the hormone *in vivo* needs further investigation.

Haynes & Berthet (459) have found that a rapid and specific activation of phosphorylase in adrenal tissue slices is caused by adrenocorticotropic hormone (ACTH). Perhaps, this ACTH action is brought about by the same mechanism which is assumed by Sutherland & Rall (460, 461) for the activation of phosphorylase by epinephrine and glucagon, namely, by the formation from ATP of a heat-stable cyclic dinucleotide (dianhydrodiadenylic acid) stimulating the formation of active phosphorylase in liver.

Haynes & Berthet (459) suggest that ACTH causes an increased formation of glucose-6-phosphate (and therefore of TPNH) as a result of the activation of phosphorylase. Since the rate of synthesis of corticosteroids in adrenal homogenates appears to depend upon the rate of formation of reduced TPN, the proposed mechanism would explain the stimulation of corticosteroid synthesis by ACTH.

Several steroid hormones were reported to increase liver transaminase activity (462) and to inhibit pyruvate oxidase in liver (463). Both observations help to explain the increased gluconeogenesis caused by glucocorticoids. Topper & Pesch (464, 465) have observed a profound stimulatory effect of progesterone, hydrocortisone, cortisone, and methyl testosterone upon the oxidation of galactose by rabbit liver slices. Since fructose and glucose metabolism are stimulated to a much slighter degree, these authors suggest that steroids attack galactose metabolism between the entry of galactose into the cell and its conversion to glucose-6-phosphate.

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AMINO ACID METABOLISM[1]

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We have chosen examples from more than 600 papers available to us through October 1958 to illustrate the growth in knowledge of the dyanmic aspects of amino acids. Even so, some major aspects of this subject must be sought elsewhere. The transport systems, for example, specify the availability and concentration of amino acids in cells and so are important determinants of amino acid metabolism as well as the related functions of intestinal and renal absorption. In this burgeoning area attention can be called only in passing to the development by Christensen and his co-workers (1, 2) of the nonmetabolizable α-amino-isobutyric acid to measure this transport in animals and to their discovery that the process is rapidly affected by certain hormones. The activation of amino acids for protein synthesis and the effect of analogues on this process must be found elsewhere in this volume. Neither can we detail work on the smaller molecules containing peptide bonds, but mention should be made of one broad generalization. Woolley & Merrifield (3) contrasted the variety of peptides which have similar actions in biological systems with the great structural specificity of vitamins and antimetabolites. To their list can be added the similar actions of glutathione and ophthalmic acid discovered by Cliffe & Waley (4). This surprising demotion of chemical structure as a determinant of biologic activity of peptides raises the possibility that configuration may play an important role even in these small molecules. Chemical evidence for the "denaturation" of the cyclic octapeptide, oxytocin, associated with loss of its activity supports this view (5).

Studies in amino acid metabolism have continued to play a large role in revealing the quantitative variability of enzymes in animal cells, but only the examples reported through 1954 have been reviewed (6). Adaptive changes in the amounts of enzymes have since been used to study specific protein synthesis and the regulation of metabolic processes. Evidence is now accumulating that this phenomenon may also underlie the differentiation of

[1] The following abbreviations are used: AMP for adenosine monophosphate; ATP for adenosine triphosphate; DNA for deoxyribonucleic acid; DOPA for 3,4-dihydroxyphenylalanine; DPN for diphosphopyridine nucleotide; DPNH for diphosphopyridine nucleotide (reduced form); FAD for flavin-adenine-dinucleotide; GSH for reduced glutathione; GSSG for oxidized glutathione; RNA for ribonucleic acid; TPN for triphosphopyridine nucleotide; TPNH for triphosphopyridine nucleotide (reduced form).

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the cell. Some of the better defined examples of enzymatic adaptation drawn from recent studies in amino acid metabolism in animals have therefore been collected to illustrate this rapidly developing field.

QUANTITATIVE ALTERATION OF SPECIFIC PROTEINS IN ANIMAL CELLS

It is no longer necessary to warn against identifying the whole phenomenon of enzymatic adaptation with substrate induction. Compounds which show no affinity for the enzyme are often effective stimuli of adaptation in both animals and microorganisms. α-Methyl-DL-tryptophan, which is not a substrate and is a very poor inhibitor of the liver tryptophan pyrrolase (formerly called the tryptophan peroxidase-oxidase), induced the formation of this enzyme in intact rats (7) and also in adrenalectomized rats (8). The latter finding eliminated possible action through adrenal stress. The production of adaptative changes in enzyme levels by their products and by hormones, as well as by their substrates and analogues, is striking proof that a substance need not be acted on by an enzyme to cause adaptation of that enzyme.

Substrate-induced enzyme changes.—The prime example of this kind of adaptation is the liver tryptophan pyrrolase. Work on this system through 1957 was reviewed by Knox (9). The studies reviewed included the demonstration by Gros and associates (10) that the adaptive increase of the enzyme was accompanied by a preferential incorporation of ^{14}C-valine into the liver fraction containing the enzyme, suggesting that new protein synthesis had occurred, and the demonstration by Price & Dietrich (11) that large increases of enzyme could be induced within a few hours in perfused liver by tryptophan (if a complete amino acid mixture was perfused). Chytil (12) reported that the adaptive increase of tryptophan pyrrolase was not affected by the administration of 4-aminopteroylglutamic acid (aminopterin), and Posnanskaya (13) that the adaptation was not changed in biotin-deficient rats. Preliminary studies have been reported of the stimulation of RNA synthesis which occurs with and after the induction of the enzyme by tryptophan (14, 15, 16).

The changes in enzyme level in a variety of physiological situations were also given in the review cited. In general, the enzyme level was low when body protein was being deposited and high when body protein was being degraded, as if the amount of tryptophan to be metabolized were one factor controlling the enzyme level. The elevation in concentration of the enzyme discovered by Schor & Frieden (17) in alloxan diabetic rats, which occurred in association with the breakdown of body protein, can be added to the generalization stated above. A paradoxical effect of insulin, which increased the enzyme level of nondiabetic animals, even in the absence of adrenals, was also observed. This effect of insulin should be considered for the present, as a hormone-induced adaptation.

Additional examples of substrate induction are the large increase in threonine dehydrase levels produced by threonine administered *in vivo* or

in the perfused liver of rats and mice [Sayre, Jensen & Greenberg (18)], and the increases in tyrosine and tryptophan transaminases effected by administration of their respective substrates [Lin & Knox (19, 20)]. Both the tyrosine and tryptophan transaminases (apoenzymes) could be induced equally well in pyridoxine-deficient and normal animals, indicating that function was not essential for their induction (21).

True substrate induction of the tyrosine transaminase could only be demonstrated in adrenalectomized rats as a doubling of the enzyme level, already raised by treatment with hydrocortisone. Tyrosine administration increased the level of tyrosine transaminase only in the presence of adrenal steroids. The requirement for priming by corticoids in this case of substrate induction is an example of the dependence of adaptive responses upon the metabolic state of the animals. It is well established that a given stimulus will produce a change in enzyme amount only in certain organs, or in animals of a particular sex, species, or physiological state. Few of the essential factors in the metabolic state of the responding tissue have been defined so precisely as this requirement of the liver for adrenal corticoid pretreatment before it can respond to tyrosine.

Not all enzymes adapt in response to their substrates. Auerbach & Waisman (22) found that liver arginase, histidase, and phenylalanine-alanine transaminase were not increased 5 hr. after the administration of their respective substrates to rats. However, liver arginase is known to be increased by high-protein diets. Goswami, Robblee & McElroy (23) reported that the cysteine desulfhydrase activity in chick liver is high at hatching and declines rapidly thereafter. A simulated egg ration providing high intakes of protein and of sulfur-containing amino acids maintained the enzyme at a high level in the chick liver after hatching.

Hormone-induced enzyme changes.—Various glucocorticoids administered to animals or released from the adrenals by stress increase the level of liver tryptophan pyrrolase in animals. Evidence that the corticoids increase the enzyme level independently of any effect on the substrate concentration in the liver is of fundamental importance for recognizing hormones as inducers of enzymes. Knox (9) reported that an elevation of the free tryptophan level in liver was easily demonstrable during tryptophan induction, but that no increase in free liver tryptophan levels occurred during hydrocortisone induction. Tryptophan metabolites were not excreted in the urine when pyridoxine-deficient rats were given hydrocortisone (8). The elevation of the tryptophan pyrrolase by hydrocortisone appears to be an example of "gratuitous" induction, an increase of an enzyme without extra function to perform. The main conclusion, that the hormones can act independently in inducing enzyme formation, is of sufficient general importance to invite more extensive documentation of this role.

In contrast to the pattern of induction of tryptophan pyrrolase, independently by its substrate and by certain hormones, the induction of the tyrosine transaminase results primarily from adrenal hormone release caused by

the stress of substrate injection as shown by the elimination of the response following adrenalectomy. The hormone is a sufficient inducer by itself; the substrate is effective as an inducer only in the presence of the hormone. Adrenal hormone induction of the tyrosine transaminase during stress is possibly the mechanism for the defect in tyrosine oxidation observed by McElroy, Anderson & Gray (24, 25) in rat liver homogenates prepared several hours after tourniquet injury to the animals. The oxidation of tyrosine, via transamination and oxidation of p-hydroxyphenylpyruvate, was inhibited in these preparations and p-hydroxyphenylpyruvate accumulated. A normal rate of oxidation was obtained if additional ascorbic acid was added to the system. Since excess p-hydroxyphenylpyruvate inhibits its own oxidation and increases the requirement for ascorbic acid in this system (26), an increased rate of p-hydroxyphenylpyruvate formation from a high induced level of tyrosine transaminase could account for the observations made on the injured rats.

Increases of the glutamic-aspartic and glutamic-alanine transaminases in rat liver after cortisone treatment were reported by Gavosto, Pileri & Brusca (28). They suggested that this treatment promoted gluconeogenesis. The increase in transaminases as a mechanism for increased gluconeogenesis is supported by the observation of Borel, Ryser & Frei (29) that a high-protein diet, which also promotes gluconeogenesis, results in a doubling of the two glutamic transaminase levels in rat liver. Rosen and his co-workers (27) found that the hydrocortisone effect on glutamic-aspartic transaminase was minimal, but that the glutamic-alanine transaminase level was doubled by the second day of treatment. This was a slow increase in comparison with the increase of tyrosine transaminase to its maximum level in 5 hr. Continued treatment for a week produced levels of glutamic-alanine transaminase up to five times the normal. As with the tyrosine transaminase, pyridoxine deficiency did not affect the adaptation.

The results of Rosen and his associates differ from an earlier report by Brin & McKee (30) that the glutamic-aspartic transaminase of liver was increased more by cortisone than was the glutamic-pyruvate alanine transaminase. In the earlier study glutamic-aspartic transaminase in the intestine was also increased by cortisone, and severe stress stimuli such as x-irradiation, nitrogen mustard, and fasting increased both transaminases in the liver by more than 50 per cent. Such differences as are seen in these studies, with one or both transaminases increased by similar stimuli, are often to be explained by subtle differences in the metabolic state of the animals used and in the stimuli given.

Earlier reports that liver arginase was increased by cortisone treatment (31) or by cold stress (32) have been borne out by a thorough study of Bach, Carter & Killip (33). The liver arginase level decreased in two weeks after adrenalectomy to one-fifth the normal level. Cortisone treatment restored the activity to normal and in intact animals raised it half again above normal level.

Additional instances of the hormonal control of enzyme concentration are the increases in amino acid activating enzymes in rat uterus produced by estradiol (34) and the depression of the leucine dipeptidase level in rat parathyroids by dihydrotachysterol treatment. The latter enzyme was also elevated by a diet stimulating parathyroid activity, which suggested to Pearse & Tremblay (35) that the enzyme is possibly active in forming parathyroid hormone from a precursor. Hicks & West (36) deduced from the low levels of histamine and 5-hydroxytryptophan in the tissues of cortisone-treated rats that this hormone lowers the levels of histidine and 5-hydroxytryptophan decarboxylases, and thus suppresses formation of the amines.

Product-depression of enzyme level.—Two instances have been observed in animals of the phenomenon called "product repression of enzyme synthesis" in microbiological systems. De Mars (37) described the depression by glutamine of the glutamine synthetase level in HeLa cell cultures. The level of the enzyme, measured by glutamyl transfer to hydroxylamine in the presence of the necessary cofactors, was so depressed in cells grown in glutamine that the cells were substantially without enzyme and died if then put into media containing glutamic acid but no glutamine. Given a low initial enzyme level, growth in the latter medium resulted in a fifteenfold increase in the enzyme. Auerbach & Waisman (38) observed that tyrosine administration to rats decreased the level of liver phenylalanine hydroxylase to one-third of normal within 5 hr. This finding necessitates revision of a conclusion reached by Moss & Schoenheimer (39) in their original experiments that showed that deuterium-labeled phenylalanine is converted to tyrosine in the rat. They believed that this reaction continued irrespective of the tyrosine supplied in the diet. The reaction was probably resumed soon after each feeding during their three-day experimental periods. By feeding a diet high in tyrosine to young rats, Auerbach, Waisman & Wycoff (40) were able to depress the level of phenylalanine hydroxylase to one-tenth the normal. By feeding a diet also rich in phenylalanine, which does not affect the level of this enzyme, they produced a condition somewhat analogous to phenylketonuria in man. There was some evidence that rats maintained for several weeks on this regimen had a decreased learning ability.

Change in enzyme levels during development.—The pattern of enzymes in embryonic and postembryonic tissues (6, 41) is continually changing in an orderly way, and some of these changes are dramatic. Kenney, Reem & Kretchmer (42) reported that phenylalanine hydroxylase is absent from the livers of fetal and newborn rats, pigs, rabbits, and infants. A few days after birth the enzyme concentration in rats attains the adult level. The missing protein component was shown to be the phenylalanine hydroxylase itself, which is also missing from the livers of phenylketonuric patients, and not one of the ancillary proteins or coenzymes. Kretchmer and his co-workers (43, 44) reported that the tyrosine oxidizing system of liver first appears a few hours after the birth of infants and rats. Although the tyrosine

transaminase reaction was not specifically measured, it would appear from these experiments that this is the limiting step in tyrosine oxidation at birth. Nemeth & Nachmias (45) reported that the tryptophan pyrrolase activity appears only in low concentrations, very late in gestation, in guinea pig fetal liver. It then increases rapidly to the adult level 24 hr. after birth. No adaptive enzyme increase could be demonstrated by tryptophan administration before birth, but within 24 hr. the enzyme became normally responsive to tryptophan injection. Nataf & Sfez (46) reported that the fetal kidneys of guinea pigs and rats are devoid of arginase. The kidney arginase appears shortly after birth and reaches adult levels in 10 to 30 days. What mechanisms control the appearance of these enzymes immediately after birth is not known. It is possible that the same mechanisms also control differentiation at earlier stages in development, where equally dramatic alterations in enzyme patterns of the cells occur. The possibility remains to be explored that some of the inducers identified above bring about the enzyme changes during development.

Glutathione concentration changes.—Enough instances of alteration in glutathione (GSH) concentration in tissues have accumulated to require some explanation of the causes and effects of these changes. Recognition of the changes as adaptive phenomena may facilitate their explanation. The following instances can be added to those reviewed in the recent Biochemical Society symposium on glutathione (47). The nonprotein sulfhydryl groups of mouse and rat liver, 90 per cent of which is GSH, undergo a nearly twofold diurnal change from a low evening level to a high morning level (48). The previously reported decreases after trauma and after cold treatment were reconfirmed in view of this diurnal variation in the control animals. Vitamin E deficiency increased the tissue GSH concentration in rabbits (49). The liver GSH level was decreased in vitamin B_{12}-deficient mice more readily than in normal controls by protein-free or low-methionine diets (50). A study of the effect of the pituitary on the localization and turnover of GSH in rat testes, liver, and adrenal cortex (51, 52) revealed an increase in adrenal GSH concentration along with the cortical hypertrophy caused by corticotropin treatment, and a diminished adrenal GSH turnover after hypophysectomy; there were no other significant changes in GSH concentration that could be attributed to the pituitary. In addition to the other stressful stimuli which are already known to decrease the concentration of free sulfhydryl groups in rat liver, β-naphthylamine and *p*-dimethylaminoazobenzene also have this effect (53). The levels returned toward normal when tumors appeared after 20 weeks' administration of the carcinogen. Diametrically opposed results were reported by Fiala (54) in the same sort of experiment. The liver nonprotein sulfhydryl groups were said to increase during early treatment with an azo dye and to remain high until they decreased when tumors appeared (after about 200 days).

GSH and methionine were the major nonprotein sulfur compounds found in unfertilized sea urchin eggs by Nakano & Monroy (55). Labeled

GSH rapidly disappeared after fertilization though the amount of GSH remained constant during the development of the eggs. The turnover of methionine was considerably less rapid.

TYPE REACTIONS OF AMINO ACIDS

Reactions of pyridoxal phosphate.—The mechanism of pyridoxal phosphate action, especially the question of metal participation in the enzyme reactions, has continued to attract attention. Bergel, Bray & Harrap (56) showed that pyridoxal phosphate and vanadium formed an efficient, nonenzymic model of the cysteine desulfhydrase reaction; the model system was active at pH 6 and room temperature. Fe^{3+} tripled the activity of a purified glutamic-aspartic transaminase from green beans, and metal chelators slightly inhibited it (57). However, Turner (58) called attention to the fact that apparent activation of this transaminase by magnesium, for example, could be explained by the nonenzymic decarboxylation of oxaloacetic acid, catalyzed by the metal. The search for metal functions in pyridoxalphosphate-requiring enzymes would appear to have been more successful with the decarboxylases than with the transaminases. Eggelston (59) described the activation of lysine, ornithine, and glutamic acid decarboxylases from *Escherichia coli* and *Clostridium welchii* by a series of divalent cations. Brown (60) gave evidence for the participation of Fe^{++} along with pyridoxal phosphate in a reaction leading to the synthesis of δ-aminolevulinic acid by chicken erythrocyte preparations, possibly at the stage of α-amino-β-ketoadipic acid formation. In no instance does the participation of a metal in an enzymic reaction of pyridoxal phosphate appear to be well enough documented to establish a mechanism resembling those of the familiar model systems. Snell has reviewed this subject and the relation of vitamin B_6 structure to its catalytic activity (61).

Investigation of the mode of action of pyridoxal phosphate has been stimulated by the antivitamin B_6 and antituberculous activities of isoniazid. From a study of the inhibition of pig heart glutamic-alanine transaminase by five cyclic hydrazides related to isoniazid, Hicks & Cymerman-Craig (62) concluded that there is no relation between the antituberculous activity and effect on this enzyme. The compounds tested were all equally inhibitory, which suggested that their action was by hydrazone formation with the coenzyme. The transaminase activities of tubercle bacilli, sensitive or resistant to isoniazid, were qualitatively the same, making it unlikely that the bactericidal action of isoniazid results from effects on these enzymes (63). Gonnard (64) observed that the isonicotinylhydrazone of pyridoxal phosphate is more active than pyridoxine phosphate itself as a cofactor for kidney DOPA-decarboxylase.

Transaminations.—α-Ketoglutaramic acid, the presumed product of the enzymic transamination of glutamine, is hydrolyzed by the liver system in which this reaction was first studied and, hence, was not found among the reaction products. Monder & Meister (65) have now demonstrated this com-

pound to be the product of the same reaction in *Neurospora*. The stability of the amides in the *Neurospora* preparation also enabled them to demonstrate a glutamine-asparagine transaminase: Glutamine + α-ketosuccinamic acid → α-Ketoglutaramic acid + asparagine. Goldstein *et al.* (66) have studied the transamination of glutamine in the guinea pig.

The tendency to identify transaminases in general with the two classical glutamic-aspartic and glutamic-alanine enzymes, fostered by recent clinical use of "the transaminase" levels in serum and by the fact that few of the host of other transaminases have been purified, is nevertheless losing ground. The reason can be seen in the list of the new reactions, if not new enzymes, reported this year: glutamic-β-amino*iso*butyric and glutamic-β-alanine in pig kidney (67); glutamic-amino-malonic in silkworm larvae and rat heart and liver (68); glutamic-glycine in silkworm (69), glutamic-δ-aminolevulinic in *Corynebacterium diphtheriae* (70) and in mammalian tissues (71); glutamic-γ-aminobutyric in beef brain (72); transamination of D-, L- and *meso*-diaminopimelic acid and of D- and L-lysine in bacteria (73); an enzyme in *Rhodospirillum rubrum* active on D-glutamic in the absence of racemase activity (74); and enzymes active on phenylalanine, tyrosine, tryptophan, and histidine in rat liver (75). Two separate transaminases, active with α-ketoglutaric or pyruvic acid exist for each of the last named four amino acids. Transamination of hydroxyaspartic acids (to oxaloglycolic) was used to show that its spatial configuration was that of *meso*-tartaric acid (76). Surveys were also reported of the various transaminations detectable in animal tissues (77), in *Fusarium lycopersici* (78) and in *R. rubrum* (74), as well as additional sources or preparations of the two classical enzymes (79, 80). A general spectrophotometric method for assaying aromatic amino acid transaminations, depending upon the absorption of the enol borate complex of the keto acids, was described by Lin and his co-workers (75).

Decarboxylations.—$^{14}CO_2$ was used by Koppelman, Mandeles & Hanke (81) to measure the equilibrium constants for the decarboxylations of lysine and glutamate. As expected, both are very large, but that for lysine is ten times that for glutamate.

An aspartic acid decarboxylase in acetone powders of *Nocardia globerula* was described by Crawford (82). Since this preparation was inactive toward other amino acids, this system could be used for aspartic acid assays.

A decarboxylase in bacteria active upon leucine, isoleucine, valine, and α-aminobutyric acid was described (83, 84). Formation of the enzyme was induced by any of the substrates. Nonsubstrate compounds which induced or which inhibited induction were found, giving additional support for the view that enzyme inducers act at a different site from the substrate.

The expected activity of pyridoxal phosphate in diaminopimelic acid decarboxylase could be demonstrated only in the cells of one strain of *Bacillus sphaericus asporogenous,* and this system was atypical in that it required more than 0.5 millimoles of the cofactor to saturate it (85).

Buzard & Nytch (86) reported a decrease in 5-hydroxytryptophan decarboxylase activity in the kidney of pyridoxine-deficient rats. It was restored in part by addition of pyridoxal phosphate *in vitro*.

Racemization.—*Bacillus subtilis,* which has a racemase for alanine but not for leucine or valine, reductively aminated the appropriate keto acids with ammonia to form DL-alanine, L-leucine, and L-valine; D-leucine and D-valine were not produced (87). A lysine racemase was demonstrated in *Proteus vulgaris* by Huang, Kita & Davisson (88).

Glutaminases—Four types of reactions which can give ammonia from glutamine are sometimes attributed to "glutaminases": hydrolysis or transfer of γ-glutamyl to an acceptor (γ-glutamyl transferase); a phosphate-dependent hydrolysis (glutaminase I); glutamine transamination dependent on the presence of an α-keto acid (glutaminase II, but preferably called glutamine transaminase); and hydrolysis dependent on ATP and Mg^{++} (glutamine-synthesizing enzyme). The last three reactions in guinea pig tissues were studied by Goldstein and co-workers (89, 90, 91) as part of their investigation of the elevation of glutaminase in kidney during acidosis (91a).

The mechanism of the synthesis of glutamine from glutamate, ammonia, and ATP was studied by Varner, Slocum & Webster (92), who showed that, just as in the phosphorolysis of glutamine, ^{18}O was transferred from arsenate during arsenolysis. They concluded that in both reactions an enzyme-bound intermediate was formed. Wieland *et al.* (93) provided even more detailed evidence for the nature of the intermediate of the glutamine synthetase reaction of peas, viz. glutamic acid was γ-glutamyl bound through a covalent linkage to the protein by means of phosphate, probably to a sulfhydryl group. Sayre & Roberts (94) came to similar conclusions in a study of the phosphate-activated glutaminase prepared from dog kidney. Inhibition by sulfhydryl active reagents was reversed by cysteine; ammonia inhibition was reversed by glutamine; and bromsulfalein inhibition (which identifies this particular enzyme) was reversed by phosphate.

AMINO ACID OXIDATION

The first direct evidence that amino acid oxidation occurs through the long postulated α-imino acid intermediate was presented by Pitt (95). The α-imino acid and its eneamine tautomer can be considered as analogues, respectively, of the enol and keto forms of an α-keto acid. In the presence of an excess of enol-keto tautomerase, amino acid oxidation gave rise to the temporary accumulation of an intermediate identified as the eneamine derivative. It was assumed to arise from the more labile and hitherto unknown α-imino acid by the action of the tautomerase.

The full description appeared of Radhakrishnan & Meister's (96) reversal of the D-amino acid oxidase reaction. This had previously been achieved by a coupled oxidation with another amino acid to bring about an effective transamination between two amino acids.

Comparison of the metabolism of D- and L-cystine, by administration of these compounds and their derivatives to rats, with analysis of the urine excretory products (97), suggested that the L-form was decarboxylated and oxidized to taurine derivatives, while the D-form was primarily attacked at the sulfur atom with preservation of the carbon chain. A reason for the disappearance of other recognizable derivatives of the D-form was later found in the observation (98) that D-cysteine sulfinic acid disappeared (to pyruvate) by oxidative deamination catalyzed by a kidney enzyme different from the usual D-amino acid oxidase and identified as the D-aspartic oxidase which had been described earlier by Still and his co-authors (99). Rocca & Ghiretti (100) described a new D-amino acid oxidase, specific for D-glutamic and D-aspartic acids, which they purified substantially from the hepatopancreas of octopus. It contained flavin-adenine-dinucleotide (FAD), which was resolved, and it catalyzed the classical type of reaction.

Yoshimoto (101) described the competitive inhibition of D-amino acid oxidase by D-lysine, and reported the absence of inhibition by ε-acyl-D-lysines. Murachi & Tashiro (102) showed that L-lysine did not inhibit the enzyme, and that the affinity of D-lysine for D-amino acid oxidase was equal to that of D-alanine. D-Lysine was not oxidized because of "the low reactivity of the D-lysine-enzyme complex."

Kubo et al. (103) crystallized glutamic acid dehydrogenase from human liver and D-amino acid oxidase from pork liver. The enzymes contained zinc and iron respectively. Adelstein & Vallee (104) found 2 to 4 gm. atoms of zinc per mole of crystalline glutamic dehydrogenase from beef liver. Frieden (105) reported that glutamic dehydrogenase (mol. wt.~one million) was split to four monomers by either DPNH or o-phenanthroline. This recalls the isolation of L-amino acid oxidase from rat kidney by Green (106) in two equally active forms with molecular weights of 400,000 and 100,000.

An L-amino acid oxidase in turkey liver which converted 5-hydroxylysine to its α-keto acid was described by Boulanger, Bertrand & Osteux (107, 108). The product underwent ring closure and could then be reduced by H_2 and Pt to 5-hydroxypipecolic acid, a compound already described as occurring naturally in dates. The existence of two types of L-amino acid oxidase in *P. vulgaris,* described long ago by Stumpf & Green (110) as a stable system oxidizing most of the monocarboxylic amino acids and a labile system oxidizing the dicarboxylic and diamino acids, was reaffirmed by Clarke (109).

PHENYLALANINE

Two protein components are required for the activity of the phenylalanine hydroxylase system of liver. Only one of these is the phenylalanine hydroxylase, and it has been mentioned that this component is missing from the liver of phenylketonuric patients and from the liver of newborn animals. The second component occurs in a large number of tissues and has some ancillary function related to the unknown cofactor of the system that Kaufman (111) described. Kaufman discovered that this cofactor can be replaced by tetrahydrofolic acid (112) which, however, does not function

catalytically as does the natural cofactor. Attempts to ascribe the mental deficiency of phenylketonuria patients to various enzymic inhibitions by the accumulated metabolites led to the observation that $0.02\ M$ phenylacetate and p-hydroxyphenylacetate inhibited brain glutamic acid decarboxylase (113). Other metabolites were less inhibitory. Similar inhibitions of DOPA-decarboxylase and 5-hydroxytryptophan decarboxylase were previously described (114, 115).

Following the observation that phenylalanine and β-phenylserine give rise to benzoic acid in man and rats, Bruns & Fiedler (116) investigated the mechanism of these reactions. From rat liver and kidney they prepared an enzyme, phenylserine aldolase, which specifically split L-*threo*-β-phenylserine to benzaldehyde and glycine. The reaction was reversible (forming some of the *erythro* compound) and required pyridoxal phosphate. The new enzyme was not the same as a threonine or serine aldolase.

<div align="center">TYROSINE</div>

Tyrosine oxidation with ring opening.—The postulated absence of homo-gentisate oxidase from the livers of alkaptonuria patients, which was the basis of Sir Archibald Garrod's fifty-year-old concept of "inborn errors of metabolism" (117), was proved by La Du and his associates (118). Enzymic studies on alkaptonuric liver removed at operation showed this enzyme ac-tivity to be missing and the other enzymes of tyrosine oxidation to be present. An interesting point was the normal activity of maleylacetoacetate isomerase which catalyzes the step immediately following homogentisate oxidation and which could never have met its substrate in the alkaptonuric liver. The presence of substrate was apparently not essential for the elabora-tion of this enzyme. The absence of the homogentisate oxidase accounts for nearly all metabolic observations which have been made on this disease, with the notable exception of Dakin's claim that alkaptonuric patients as well as normal animals can oxidize the ring of phenylalanine derivatives blocked in the *para*-position. This claim long prevented the acceptance of the basic concepts of biochemical genetics. Pirrung, Gottesman & Crandall (119) and Ichihara, Umezawa & Sakamoto (120) now have disposed of this objection by demonstrating that this reaction cannot occur even in normal tissues. The scientific history of alkaptonuria as the prototype of inborn errors of metabolism was reviewed by Knox (121).

The pathway of tyrosol formation by *Saccharomyces cerevisiae* was deduced from studies on cell extracts by Sentheshanmuganathan & Elsden (122). It occurs by transamination of tyrosine with α-ketoglutarate to form p-hydroxyphenylpyruvate. The latter is decarboxylated to p-hydroxyphenyl-acetaldehyde and then reduced by DPNH and alcohol dehydrogenase to tyrosol.

Tyrosinase and melanin formation.—Krueger (123) reported that ascorbic acid and also DPNH eliminated the induction period of tyrosinase and in-creased its rate of oxidation of monophenols. He called attention to the similarity between this and the requirement for reducing agents in other

enzymic hydroxylations. Scharf & Dawson (124) demonstrated that an action of ascorbic acid later in the reaction (its reported reversal of the inactivation of tyrosinase during oxidation of catechol) was illusory. Brown & Ward (125) obtained three fractions of mammalian tyrosinase of different purity, all with similar activities on tyrosine and DOPA.

In a review of human albinism, Knox (126) discussed the circumstantial nature of the evidence for the commonly accepted view that melanin is formed in man from tyrosine and DOPA. Fox & Burnett (127) showed that an albino mutant of *Neurospora crassa* possessed a tyrosinase which differed from the wild type enzyme only by a lagging accumulation of DOPAchrome. The reason for this lag was not proved, though it was attributed to the time necessary for activation of a protyrosinase. Tyrosine and DOPA have been even more firmly associated with melanization in insects than they have in higher animals, yet, even here, new experiments also cast doubt on this commonly accepted role. By administration of various phenols to albino locusts, Jones & Sinclair (128) demonstrated that sclerotinization and melanization are separate processes. Protocatechuic acid and DOPA are concerned with the former process, while catechol is exclusively concerned with melanization. A role for catechol as a precursor of melanin in insects is supported by its natural occurrence in insects (129). From the silkworm, Kawase (130) isolated protocatechuic acid and demonstrated that treatment with it yielded naturally colored pupae, while tyrosine yielded abnormal purple-black colorations.

Some melanoma patients undoubtedly excrete melanogens, possibly derivatives of indole-5,6-quinone (131), but this occurs in fewer than the one-third of the patients usually claimed. Three autoxidizable phenolic compounds were isolated from such urines, and it is said that the structures will be published (132).

The occurrence of an abnormal compound in the plasma of melanoma patients was described by Riley (133). A nonenzymic oxidation of *p*-phenylenediamine occurred in the plasma of melanoma patients, but not in the the plasma of patients with other types of cancer. This oxidation could be duplicated by addition of DOPA or catechol to solutions of *p*-phenylenediamine.

Diiodotyrosine and thyroxine.—Serif & Kirkwood (134) described soluble and particulate thyroid systems which converted L-tyrosine to monoiodotyrosine. The soluble system required hydrogen peroxide, and both systems were inhibited by catalase but not by CO, which was believed to distinguish this system. The conversion of tyrosine and iodide to 3,5-diiodotyrosine by horseradish peroxidase and peroxide was described (135). Since this enzyme system is known to oxidize iodide to iodine, and iodine is known to react with tyrosine, this result is not surprising. There is a danger of the same side reaction, perhaps catalyzed by methemoglobin, in all studies of the physiological iodination system.

Following the observation that 3′,5′-dimethyl analogues of thyroxine are more active than thyroxine itself, Kharasch & Saha (136) synthesized an

analogue with *t*-butyl groups, which are even more strongly electron releasing, in the 3′ and 5′ positions. They hoped to produce a sterically hindered analogue which would competitively inhibit thyroxine action. Assays of the compound, to be accomplished by the stimulation of glucose oxidation in yeast by 10^{-2} to 10^{-10} M thyroxine (137), are not yet available. However, the success of this approach was anticipated in the choice of the name "Hinderin-A" for the first derivative.

Tata (138) investigated the degradation of L-thyroxine and triiodo-L-thyronine in extracts of rat brain and muscle. In both tissues the deiodinase reaction was greater than deamination. The deiodination occurred in the soluble fractions of both tissues, and all of the iodine of thyroxine was converted to iodide without the intermediate formation of triiodothyronine. Wilkinson (139) reported that tri- and tetraiodothyroacetic acids were deiodinated less than the related thyroxine derivatives. Stanbury & Morris (140) described a liver system which catalyzes the stepwise deiodination of diiodotyrosine. This reaction occurs in a microsomal system which requires TPNH. The kinetics of diiodotyrosine metabolism in normal human subjects was studied by Ruegamer & Chodos (141).

Epinephrine metabolism.—The long uncertainty about the role of amine oxidase in terminating the activity of epinephrine in the body has been decisively answered by Axelrod and his collaborators (142, 143, 144). The major inactivation of epinephrine in man and rat occurs by O-methylation to *m*-methoxyepinephrine (metanephrine). Monoamine oxidase acts later to deaminate this compound to 3-methoxy-4-hydroxymandelic acid. Norepinephrine follows the analogous pathway. These reactions were demonstrated with a soluble and Mg-requiring system from brain which utilized S-methyladenosylmethionine as the source of the methyl groups, and also by degradation studies with labeled compounds in the intact animals. The failure of iproniazid, a monoamine oxidase inhibitor, to prolong epinephrine action is explained by these results.

TRYPTOPHAN

A dephosphorylated derivative of an intermediate in the biosynthesis of tryptophan by *E. coli.*, already proposed by Yanofsky (146), was accumulated by washed cell suspensions of two strains of *Aerobacter aerogenes* requiring either indole or tryptophan for growth. It was isolated as a hydrazone by Gibson, Doy & Segall (145), who deduced it to be 1-N-1-deoxyribulosylanthranilic acid. Concentrates of the compound supported the growth of mutants able to utilize anthranilic acid. The pathway in yeast may differ, since Parks & Douglas (147) have tentatively identified N-fructosylanthranilic acid as a compound accumulated by a tryptophan auxotroph of *Saccharomyces*.

Yanofsky & Rachmeler (148) excluded free indole from the pathway of tryptophan biosynthesis in *N. crassa*, which occurs by the following exchange: indoleglycerylphosphate + tryptophan synthetase ⟶ indole-tryptophan synthetase + triosephosphate; indole-tryptophan synthetase + L-

serine $\longrightarrow$ L-tryptophan + tryptophan synthetase. Suskind & Kurek (149) prepared an active tryptophan synthetase from inactive crude extracts of a tryptophan auxotroph of *N. crassa.* A metal inhibitor was removed during the purification procedure. The inhibitor was present in extracts of both wild type and mutant strains, but the mutant enzyme was much more sensitive to it.

The enzyme responsible for the conversion of tryptophan to L-formylkynurenine was long thought to catalyze a coupled reaction involving oxidation and peroxidation: one mole of oxygen is used in the reaction and catalase inhibition may be overcome by a peroxide generating system. Two papers have clarified the nature of this reaction. Hayaishi *et al.* (150) demonstrated that molecular oxygen is incorporated into the formyl and carbonyl groups of formylkynurenine. Knox & Tanaka (151), in a preliminary report, showed that the enzyme contains a porphyrin nucleus and that the active form of the enzyme is the ferrous porphyrin protein. Peroxide acts only to reduce the inactive ferric enzyme to the active ferrous form which then functions as an oxygen-transferring enzyme. Its designation as the "tryptophan peroxidase-oxidase" (TPO) is now clearly a misnomer. It will be referred to henceforth by the name Kotake (see 152) had originally assigned it, "tryptophan pyrrolase."

Using tritium-labeled DL-kynurenine, Hankes & Segal (153) showed that its conversion to nicotinic acid definitely takes place in the rat. Saran (154) described methods for the partial purification and some properties of a kynureninase from *N. crassa.* He found many differences in the properties of this enzyme as compared with those described by Jakoby & Bonner (155) for a kynureninase prepared from the same organism.

Compounds related to tryptophan.—An interesting study of the indoleacetic acid oxidase of pineapple by Gortner & Kent has appeared (156). Pineapple extracts contain *p*-coumaric acid, which serves as a cofactor, and ferulic acid, which is a powerful inhibitor of the activated enzyme. A *Pseudomonas* capable of growth on indoleacetic acid was isolated by Proctor (157). Organisms grown on this compound were able to oxidize 3-methylindole, 3-hydroxyindole, salicylic acid, and pyrocatechol without any lag. Pyrocatechol, 3-methylindole, and salicylic acid were isolated from the cultures. These observations suggest a new route for the breakdown of the indole nucleus.

The oxidation of a series of ω-(3-indolyl)-alkanecarboxylic acids to indoleacetic or indolepropionic acid by pea and wheat tissues was reported by Fawcett *et al.* (158).

NICOTINE

Studies on the biosynthesis of nicotine (159, 160, 161) showed that its pyrrolidine ring may arise from ornithine, putrescine, proline, or glutamic acid. None of these contributes to formation of any portion of the pyridine ring. Both 2-^{14}C-ornithine and 2-^{14}C-glutamic acid gave rise to a symmetrical labeling in the 2- and 5-positions of the pyrrolidine ring, which argues

strongly for a symmetrical intermediate in this biosynthetic pathway. Grimshaw & Marion (162) discussed the problem of the biosynthesis of the pyridine ring in plants. As yet only the negative evidence is available that it is not formed from tryptophan or any other compounds tested.

Frankenburg & Vaitekunas (163) described a number of degradation products of nicotine that were formed when this compound was incubated with mixed cultures of bacteria derived from washings of tobacco seeds. Wada & Yamasaki (164) had isolated one of these compounds, 3-succinoyl-pyridine, from a pure culture of *Pseudomanas* grown on nicotine. More recently, Wada (165) isolated two additional compounds, pseudo-oxynicotine [3-(4-methylaminobutyroyl)-pyridine] and 3-succinoyl-6-hydroxypyridine. On the basis of these compounds and the evidence provided by simultaneous induction experiments, he postulated a pathway for nicotine degradation by these organisms which involves oxidation of the pyrrolidine ring followed by pyridine oxidation at position-6. The isolation of 3-succinoyl-6-hydroxypyridine was also reported by Hylin (166). The closely related compounds, anabasine and nornicotine, were catabolized by analogous routes (165), although hydroxylation of the pyridine ring appeared to precede hydrogenation of the pyrrolidine ring. Knowledge of the pathway of nicotine degradation is still fragmentary, but it seems clear that the initial steps are concerned with the breakdown of the pyrrolidine ring together with hydroxylation of the pyridine moiety. It is probable that the further steps will involve 6-hydroxynicotinic acid and its degradation according to the pathway established by Behrman & Stanier (167), although the isolation of γ-aminobutyric acid from a culture of a bacterium that oxidizes nicotine must still be accounted for (168). McKennis et al. (169) isolated γ-(3-pyridyl)-γ-methylaminobutyric acid from the urine of dogs fed nicotine, which must have been formed by a pyrrolidine ring cleavage alternative to that discussed above.

HISTIDINE

In support of the scheme presented in last year's review on the formation of the histidine precursor, imidazole glycerolphosphate [4-(3'-phospho-1'-glyceryl) imidazole], from ATP, ribose phosphate, and glutamine, Moyed (170) has now reported that the compound III, postulated to be the N-1-quaternary salt, 1-(5'-phospho-1'-ribosyl)-AMP, contains, in fact, two moles of pentose. Compound III arose via the condensation of 5-phosphoribosyl-1-pyrophospate with AMP, and decomposed to 5-amino-1-(5'-phospho-1'-ribosyl)-imidazole-4-carboxamide plus an unknown compound which was acted upon by another protein fraction to yield the imidazole glycerophosphate.

4-Imidazolone-5-propionic acid has been postulated for some time to be in the pathway of histidine degradation immediately following urocanic acid. Evidence for this compound as an intermediate has now been provided in beef liver (171) and in *A. aerogenes* (172). It is a labile compound, enzymatically convertible to L-α-formamidinoglutaric acid (α-formamido-

L-glutamic acid). Nonenzymatic hydrolysis occurs readily, leading to L- and D-formylisoglutamine. Neither of these compounds was degraded enzymatically. Thus the long controversy over the formylisoglutamine [2-(formylamino)-glutaramic acid] pathway versus the formamidoglutamic acid pathway seems to be resolved in favor of the latter. In addition to the hydrolytic pathway of urocanic acid degradation, Suda *et al.* (173) described the isolation of 2-ketoglutaramic acid as the product of an oxidative pathway of urocanic acid degradation in cat liver. No oxygen uptake occurred in this system until the enzyme preparation was heated, a treatment postulated to inactivate one or more hydrolytic enzymes. An apparently similar pathway for the oxidative degradation of urocanic acid was found in *Pseudomonas aeruginosa* (174). 2-Ketoglutaramic, hydantoin acrylic, and succinic acids, as well as succinylmonoureide, were isolated and identified as products of the bacterial oxidation.

Rothberg & Hayaishi (175) investigated the mechanism of the enzymatic oxidation of imidazoleacetic acid with $^{18}O_2$ and an enzyme prepared from *Pseudomonas*. One mole of oxygen was consumed in the reaction, with but one atom appearing in the product. Rothberg & Hayaishi suggested that the primary step involved the oxidation of imidazoleacetic acid to 4-imidazolone-5-acetic acid (or its enol tautomer), with subsequent hydrolysis to N-formiminoaspartic acid. This reaction is then identical in type to a number of other enzymatic hydroxylations requiring oxygen and either DPNH or TPNH. Witkop & Kny (176) synthesized 4-imidazolone-5-acetic acid, the postulated intermediate in the breakdown of imidazoleacetic acid, and found that it had a half life of about 1 hr. at pH 8. Its decomposition was accelerated by a preparation of the imidazoleacetic acid oxidase. This compound is, of course, homologous to the imidazolepropionic acid previously discussed.

Claviceps purpurea synthesized ergothionine from histidine. If 2-^{14}C- or α-^{14}C-histidine was fed, ergothionine was highly labeled in the equivalent positions, thus demonstrating the unlikelihood of either ring fission or side-chain alteration (177).

Kobayashi (178) demonstrated the existence of two histamine metabolizing systems in cat liver homogenates, one producing 1-methylimidazole-4-acetic acid, the other, imidazole-4-acetic acid. Rothschild & Schayer (179) found that the major portion of 1-methyl-4-(β-aminoethyl)-imidazole, a histamine metabolite, was excreted by mice as 1-methylimidazole-4-acetic acid.

That carnosine phosphate plays no role in muscular contraction was shown by Cain *et al.* (180), who found it to be absent from turtle muscle preparations.

PROLINE AND HYDROXPROLINE

Δ'-Pyrroline-2- and -5-carboxylic acids may both be reduced to proline by separate enzymes (181). The purified enzyme from rat kidney that catalyzed the reduction of Δ'-pyrroline-2-carboxylic acid acted as well on Δ'-piperidine-

2-carboxylic acid to yield pipecolic acid. It was operative with either DPNH or TPNH. The reduction to pipecolic acid supported the belief that this reaction was part of the biosynthesis of pipecolic acid from lysine.

Adams (182) extended his work on the conversion of hydroxy-L-proline to L-glutamate by a soil bacterium. It now appears that the pathway is hydroxy-L-proline $\longrightarrow$ *allo*hydroxy-D-proline $\longrightarrow \Delta'$-4-hydroxypyrroline-2-carboxylic acid $\longrightarrow$ 2-keto-4-hydroxy-5-aminovaleric acid $\longrightarrow \alpha$-ketoglutaric acid plus NH_3 plus L-glutamic acid. A crucial fact was the finding that iodoacetic acid blocked the (first) epimerization step but still allowed the conversion of the *allo*hydroxy-D-proline to L-glutamic acid. Furthermore, the *allo*hydroxy-D-proline oxidase was sedimentable and thus physically separable from the epimerase. In studies with a mammalian system, Adams *et al.* (183) described the oxidation of hydroxy-L-proline to Δ'-pyrroline-3-hydroxy-5-carboxylic acid by kidney particles. This compound (in equilibrium with its hydrolysis product, γ-hydroxy-glutamic semialdehyde) was converted by soluble enzymes by oxidation (plus DPN) to γ-hydroxy-L-glutamic acid or by reduction (DPNH) to hydroxy-L-proline.

Evidence continues to build up that neither free hydroxyproline nor free hydroxylysine are incorporated into proteins (184, 185, 186). Wolf & Berger (186), for example, fed L-hydroxyproline-2-^{14}C to rats. The free hydroxyproline pool was diluted sixfold; thus the pool was small. After proline, together with the isotopic compound, had been fed, the dilution of the pool was about sixtyfold; thus conversion of proline to hydroxyproline was demonstrated. The hydroxyproline of newly synthesized protein, on the other hand, was diluted about 2000 times.

Gould has reported (187) the stimulation of hydroxyproline formation in guinea pigs by ascorbic acid.

Steward and his co-workers (188, 189) performed some interesting experiments on plant tissues with regard to the proline-hydroxyproline problem. They used carrot phloem explants which synthesized a metabolically inert protein rich in hydroxyproline. L-*Allo*-hydroxyproline and L-hydroxyproline inhibited growth in the system, and the inhibition was specifically overcome by L-proline.

SULFUR-CONTAINING AMINO ACIDS

The history and nature of the hereditary disease, cystinuria, were reviewed by Knox (190).

Glutathione.—The biochemistry of glutathione (GSH) was the subject of a symposium of the Biochemical Society arranged by E. M. Crook. Abstracts of the papers have appeared (47). They include a review of the alterations in tissue concentrations of GSH in addition to those mentioned in an earlier section on adaptive changes (p. 228). Ophthalmic acid, an analogue of GSH isolated from calf lens by Waley (191), was identified and confirmed by synthesis to be γ-glutamyl-α-amino-n-butyryl-glycine. Its stepwise synthesis by a system including one or both of the GSH-synthesizing enzymes was demonstrated by Cliffe & Waley (192). The synthesis in rabbit

lens, which contains no ophthalmic acid, was equally as effective as in calf lens. Analogous tripeptides could be synthesized with α-aminobutyric acid replaced by alanine or threonine. When tested on one of the most specific biological assays for GSH, the elicitation of the feeding reaction of *Hydra*, ophthalmic acid proved to be more active than GSH (4). The adduct of GSH and N-ethylmaleimide and the sulfonic acid derivative of GSH were found to inhibit the elicitation of the feeding reaction.

GSH peroxidase, which catalyzes the oxidation of GSH to the oxidized form, GSSG, by hydrogen peroxide, was identified in erythrocytes by Mills (193). When hydrogen peroxide was generated from ascorbic acid in a suspension of red cells *in vitro*, this catalyzed oxidation of GSH proved to be more effective than catalase in protecting hemoglobin against oxidation by the peroxide.

Cystathionine.—A very high concentration of L-cystathionine in human brain extracts was reported by Tallan, Moore & Stein (194). Human brains contained from 22 to 56 mg./100 gm. wet wt. in comparison with less than 1 mg./100 gm. in other tissues and in the brains of other species except the rhesus monkey and the horseshoe crab. Matsuo & Greenberg (195) crystallized an enzyme which was of high purity by the usual criteria. It cleaved cystathionine and deaminated the resulting homoserine to form cysteine, an α-keto acid, and NH_3. The enzyme contained 4 moles of pyridoxal phosphate and no detectable metals.

Methionine.—Durell, Anderson & Cantoni (196) prepared the thetin-homocysteine methylpherase from horse liver in essentially homogeneous form. The formation of methionine was irreversible, but Durell & Sturtevant (197) nevertheless obtained an estimate of the ΔF by direct calorimetry of the reaction ($-20,000$ cal./mole).

The methionine-activating enzyme of yeast which forms S-adenosylmethionine was described by Schlenk & DePalma (198) and by Mudd & Cantoni (199). In yeast grown in the presence of ethionine, Parks (200) demonstrated the accumulation of S-adenosylethionine. Transethylation with this compound was demonstrated *in vitro*. The isolation of S-ribosylmethionine from the nonenzymic hydrolysis of S-adenosylmethionine was described by Parks & Schlenk (201). They (202) also described the formation of α-amino-γ-butyrolactone from S-adenosylmethionine in *A. aerogenes*. Shapiro & Mather (203) reported that the other product in this reaction was S-methyladenosine, which was cleaved to adenine plus methylthioribose.

Shapiro (204) described the S-adenosylmethionine-homocysteine transmethylase from a number of microorganisms. The system was active with S-adenosylmethionine or S-methylmethionine in contrast to the inactivity of the liver system with these compounds. Dimethylacetothetin and dimethylpropriothetin, which were active with the liver system, were inactive in the microbial system. Maw (205) studied a number of methyl- and ethyl- sulfonium compounds in the liver transmethylase system. He found that dimethylthetin was the most effective methyl donor, and homocysteine was the most effective acceptor. S-adenosylmethionine was a better donor than methionine

for the C-28 methyl group of ergosterol (206). Stekol *et al.* (207) have compared the effectiveness of S-adenosylmethionine as a methyl donor for choline and creatinine in different complex situations *in vivo* and *in vitro*.

A possible mechanism involving S-adenosylmethionine as an intermediate for the incorporation of formaldehyde into the methyl group of methionine was discussed by Nakao & Greenberg (208) in relation to their study of a sheep liver enzyme which performed this reaction in the presence of a number of added cofactors.

Formiminoglutamic acid, derived from histidine, was excreted by rats deficient in folic acid or vitamin B_{12}, and this excretion was decreased by dietary methionine or homocysteine. Silverman & Pitney (209) concluded that the one-carbon unit was made available by a reaction involving folic acid and vitamin B_{12} and transferred as a methyl group to a derivative of methionine. Woods and his colleagues (210, 211) reported the requirement for cobalamine along with tetrahydrofolic acid in the synthesis of the methionine methyl group by *E. coli* extracts.

Sato *et al.* (212) showed that methionine is converted in plants to methionine sulfoxide and the methyl group transferred to pectin. Methylation of pectin by methionine as a means of breaking down the calcium pectate of the walls may be responsible for the reduction in time for abscission of flowers caused by injection of L-methionine (213).

E. coli mutants blocked in methionine synthesis can use methionine sulfoxide and homocysteine as well as methionine for growth, but cannot use methionine sulfone or the sulfoxamines (214). Methionine peptides were added to this list of effective compounds for *E. coli,* and methionine sulfoxide was shown to be more effective than methionine itself in antagonizing sulfanilamide inhibition (215). Selenomethionine also completely replaced methionine for a methionine-requiring *E. coli* mutant, although not all of the sulfur requirement was satisfied (216). Tuve & Williams (217) showed that in such an experiment selenomethionine was incorporated into protein.

Wiehler & Marion (218) showed that the methylation of choline to stachydrine, a compound which normally occurs in the mature alfalfa plant, can be induced in seedlings by feeding the required precursors: ornithine as a precursor of proline; methionine as a precursor of the methyl group; and folic acid and pyridoxine as cofactors.

Mercapturic Acids.—Evidence was presented by Thomson, Maw & Young (219) and by Bray & James (220) that rabbits excrete mercapturic acid derivatives of aliphatic compounds after the administration of alkyl bromides. The possible existence of acid-labile premercapturic acids, analogous to the dihydro-diol derivatives of some carcinogens, was indicated in experiments by Knight & Young (221).

Glutamic Acid

Cell-free extracts of *Clostridium tetanomorphum* catalyze a series of reversible reactions, the first step of which involves the interconversion of glutamic and β-methylaspartic acids (222, 223). The fate of the individual

carbon atoms is indicated in Figure 1. Barker, Weissbach & Smyth (224) discovered that this reversible reaction requires a coenzyme closely related to pseudovitamin B_{12}. This is the first specific enzymic function shown for any member of the B_{12} group of compounds. It will be of interest to test this compound in other systems involving carbon chain rearrangements (isoleucine-valine biosynthesis, sterol biosynthesis, p-hydroxyphenylpyruvate oxidation, etc.).

$$
\begin{array}{ccc}
\overset{5}{} \text{COOH} & & \overset{5}{} \text{COOH} \\
| & & | \\
\overset{4}{} \text{CH}_2 & & \overset{4}{} \text{HC} - \overset{3}{}\text{CH}_3 \\
| & \rightleftharpoons & | \\
\overset{3}{} \text{CH}_2 & & \overset{2}{} \text{HC} - \text{NH}_2 \\
| & & | \\
\overset{2}{} \text{HC} - \text{NH}_2 & & \overset{1}{} \text{COOH} \\
| & & \\
\overset{1}{} \text{COOH} & &
\end{array}
$$
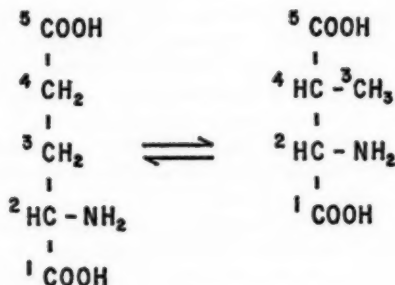
Glutamate   β-Methylaspartate

Fig. 1. The interconversion of glutamate and β-methylaspartate

Halzer & Schneider (225) demonstrated an effective transhydrogenation system using the glutamic dehydrogenase from yeast or a mammalian lactic dehydrogenase, which react with either DPN or TPN. Thus, pyruvic acid, for example, can function as a transhydrogenation catalyst in just the same way as the steroid systems recently described by Hurlock & Talalay (226).

Aspartic Acid

Krasna (227) and Englard (228) studied the stereospecificity of the aspartase reaction, using enzymes from *P. vulgaris* and from *Bacterium cadaveris*. Englard, in addition, investigated the bacterial fumarase. The authors concluded that in each of the three cases the enzyme catalyzed a stereospecific *cis*-addition.

Linko (229) fed uniformly-labeled aspartic acid to the leaves of *Convallaria majalis* (lily of the valley). The aspartic acid was decarboxylated to both α- and β-alanines, but no activity was found in azetidine-2-carboxylic acid, an unusual amino acid occurring in high concentration in this plant.

γ-Aminobutyric Acid

Studies on the metabolism of γ-aminobutyric acid in brain as well as in various organisms have shown its origin from glutamic acid (230), its transamination to succinic semialdehyde (231, 232), and its oxidation to succinic

acid (233). In addition, Noe & Nickerson (234) demonstrated that it can be derived from 2-pyrrolidone via hydrolysis in *P. aeruginosa*, while Scott & Jakoby (235), using a species of *Pseudomonas* isolated by the enrichment technique, demonstrated oxidation of pyrrolidine to form γ-aminobutyric acid. It was then converted to succinic acid by the pathway outlined above.

SERINE AND GLYCINE

Stadtman, Elliot & Tiemann (236) studied the complex glycine reductase system in *Clostridium sticklandii*. The over-all reaction may be formulated as follows:

$$NH_2 \cdot {}^{14}CH_2 \cdot COOH + R(SH)_2 + P_1 + ADP$$
$$= CH_3 \cdot {}^{14}COOH + NH_3 + RSS^- + ATP$$
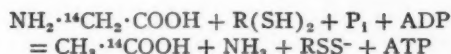
The reaction appears to be irreversible. Stickland reactions had remained intractable to study until the discovery that dithiols could serve as electron donors. Stadtman and her colleagues showed that two protein fractions are necessary for the conversion. Arsenate may replace phosphate. Because of this, it seems reasonable to infer that a phosphorylated intermediate is involved. Acetylphosphate and phosphoamidate have been excluded, however, and the likely possibility is an S-phosphoryl compound. Inhibition by fluoride indicates the participation of a metal cofactor. Labeling experiments showed that there is no mixing of the carbon atoms involved; hence this reaction is clearly distinguished from the glycine fermentations carried out by *Diplococcus glycinophilus* and *Clostridium acidi-urici* (237).

Whiteley (238) described the fermentations by *Micrococcus aerogenes* of four amino acids: serine, threonine, glutamic acid, and histidine. Serine was fermented to ammonia, carbon dioxide, hydrogen, and pyruvate. Threonine gave the same gaseous products together with α-ketobutyric acid. Glutamate was degraded through a pathway not involving mesaconic, citramalic, or α-ketoglutaric acids, but formed acetic and butyric acids, NH_3, CO_2, and H_2 as end products. Histidine was fermented through a urocanic acid pathway (see p. 237).

Mackenzie & Frisell made quantitative studies of the metabolism of dimethylglycine to serine by mitochondria from rat liver (239). Two moles of active formaldehyde were generated during the conversion of dimethylglycine to sarcosine and of sarcosine to glycine. Dac & Wriston (240) studied the effect of folic acid deficiency on the metabolism of sarcosine in the same system. Preparations from deficient rats had markedly reduced ability to form serine (85 per cent reduction), but oxygen uptake was reduced only 25 per cent. Likewise, isonicotinic acid hydrazide inhibited serine formation, but had no effect upon oxygen uptake. The authors reported quantitative data on serine and formaldehyde formation from sarcosine, but did not determine glycine. They postulated a condensation between 5-formyltetrahydrofolic acid and a pyridoxal glycine compound to form serine. White (243) calculated the rates of formation of glycine and serine from each other and of each of these amino acids from other precursors.

Delwiche & Bregoff (241) found that *Beta vulgaris* (the beet plant) does not produce betaine by the methylation of glycine. Brady & Koval (242) showed that serine may condense with palmityl-CoA (or more probably its thiosemiacetal) to form dihydrosphingosine, which is then converted to sphingosine. Serine formed carbons 1 and 2 of the product, while the palmityl moiety gave rise to carbons 3 through 18.

Two papers have appeared simultaneously on the mechanism of action of O-phosphoserine phosphatase (244, 245). The following mechanism of action was proposed: E + P-serine = E-P-serine = E-P + serine $\longrightarrow$ E + P, where E and P designate enzyme and phosphate, respectively. It was suggested (246, 247) that L-serine inhibition of O-phosphoserine phosphatase serves as a feedback mechanism that controls the formation of serine, *in vivo*. The formation of phosphoserine from 3-phosphoglyceric acid via oxidation and transamination in pea epicotyls was demonstrated by Davies (248): D-3-phosphoglycerate + DPN $\longrightarrow$ phosphohydroxypyruvate + DPNH + L-glutamic acid $\longrightarrow$ phosphoserine + α-ketoglutarate.

Watts *et al.* (252) suggested that primary hyperoxaluria results from a defect in glycine metabolism involving perhaps a pathway to oxalate via glyoxylate.

THREONINE

Elliot (249) reported the probable formation of aminoacetone from threonine by suspensions of *Staphylococcus aureus*. The likely pathway is threonine to α-amino-β-ketobutyric acid to aminoacetone + CO_2. Riley & Robinson (250) demonstrated the formation of α-aminobutyric acid from threonine by *S. aureus* under conditions of low oxygen tension. Under the same conditions the cells also formed alanine from serine. It is postulated that threonine, under these conditions, functions as a hydrogen acceptor.

The 100-fold purification of L-threonine deaminase from the rumen microorganism LCI was reported by Walker (251). Pyridoxal phosphate was required and glutathione stimulated the reaction. D-Threonine was inhibitory. L-Serine also served as a substrate.

δ-AMINOLEVULINIC ACID

Two laboratories have now provided direct evidence for the synthesis of δ-aminolevulinic acid via condensation of succinyl-CoA with a postulated pyridoxal phosphate derivative of glycine (253 to 256). Shemin and his group used extracts of the nonsulfur purple bacteria, *Rhodopseudomonas spheroides* and *Rhodospirillum rubrum,* while Neuberger used preparations of chicken erythrocytes. Other studies on steps of the synthesis include papers by Granick (257), Brown (258) and Laver *et al.* (259).

Anderson & Tone (260) found that cupric ions stimulated heme synthesis in the blood of either copper- or iron-deficient chickens. Granick & Mauzerall (261) described three soluble enzymes from erythrocytes necessary for the conversion of δ-aminolevulinic acid to coproporphyrinogen via porphobilinogen and uroporphyrinogen III. Nemeth *et al.* (262) showed the con-

version, by enzymes derived from birds and rats, of the δ-carbon to the ureido groups of guanine, uric acid, and formic acid with the concomitant production of succinate. Since the δ-carbon is derived from the α-carbon of glycine, this pathway provides a cyclic mechanism whereby succinate is regenerated and glycine is metabolized.

ISOLEUCINE AND VALINE

A number of uncertainties in the biosynthesis of isoleucine (VII, R = Et) and valine (VII, R = Me) have been cleared away (see Figure 2). It was shown previously that the two compounds immediately preceding isoleucine and valine are their respective α-keto (VI) and α,β-dihydroxy (V) analogues (263). Labeling experiments had shown the origin of valine from two molecules of pyruvate (II) (264) and had strongly suggested the
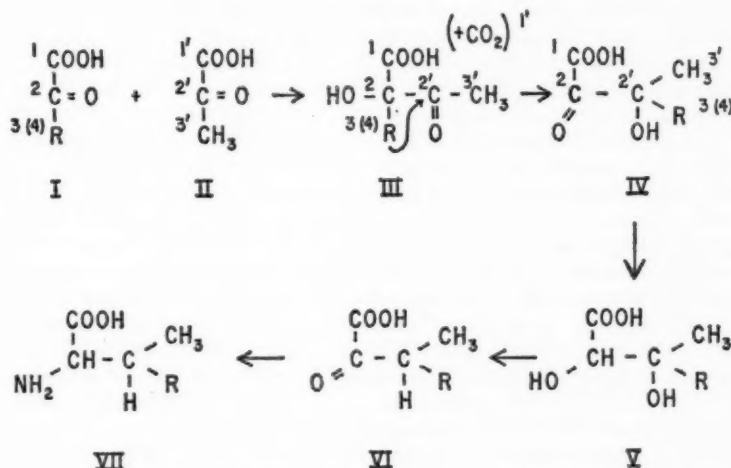
FIG. 2. The biosynthesis of valine and isoleucine

derivation of the isoleucine skeleton from threonine via α-ketobutyric acid (I, R = Et) and pyruvate (265). It was clear that rearrangement of the carbon chain of any such condensation product must occur to account for the labeling patterns in the final products, and postulated mechanisms for this have appeared (266, 266a). Umbarger et al. (267) demonstrated that α-acetolactate (III, R = Me) is a probable intermediate in valine biosynthesis by E. coli. It has now been shown that this compound is converted to α-ketoisovaleric acid (VI, R = Me) by yeast (268) and further that yeast forms it from pyruvate (269).

Wagner et al. (270) using strains of N. crassa, provided evidence on the nature of the initial condensation products and on the rearrangement and

reduction steps between these and the dihydroxy acid precursors. The conversions of III (R =Me,Et) to V (R = Me,Et) were directly demonstrated, together with the subsequent conversions to the corresponding amino acids. The synthetic α-keto-β-hydroxy rearrangement compounds (IV, R = Me, Et) are likewise converted to the amino acids via the dihydroxy acids, although their isolation has not yet been achieved.

Willson & Adelberg (271) isolated citramalic (α-methylmalic) and α,β-dimethylmalic acids from the culture medium of a *N. crassa* mutant blocked in the synthesis of both valine and isoleucine. Since these compounds could have arisen through the oxidative decarboxylation of 4-hydroxy-4-methyl-2-ketoglutaric acid (VIII) and 4-hydroxy-3,4-dimethyl-2-ketoglutaric acid (IX), the latter compounds were postulated as possible initial condensation products. This appears to have been misleading. Adelberg has since found that VIII is not converted to V (R = Me), and he confirmed the conversion of III (R = Me) to V (R = Me) (270, footnote 18). He suggests that the occurrence of citramalic and dimethylmalic acids in the *N. crassa* mutant is to be explained by the fact that this strain "is blocked prior to the formation of [III, (R = Me,Et)] and carries out an abnormal condensation, perhaps as an induced enzyme reaction."

Umbarger & Brown (272, 273) have described two kinds of product inhibition of enzymes which control the synthesis of valine and isoleucine. The formation of the enzyme which synthesizes III (R = Me) from pyruvate is depressed by valine; further, valine itself inhibits the action of the enzyme. The constitutive L-threonine deaminase, the enzyme which forms α-ketobutyrate (I, R = Et), was inhibited by L-isoleucine; the enzyme, in fact ,had an affinity for L-isoleucine 100 times greater than for L-threonine.

Rechcigl *et al.* (274) showed that the rat can utilize D-leucine for growth, Previous negative results (275) were accounted for by the presence of DL-norleucine in the diet since norleucine inhibited the utilization of D-leucine. Duda & Handler (276) studied the metabolism of ^{15}N-labeled L- and D-leucine in rats. The major pathway of D-leucine metabolism was by oxidative deamination, while L-leucine transferred its amino group to glutamate.

Webb (277) suggested a pathway in *A. aerogenes* for valine catabolism via α-ketoisovaleric acid to isobutyryl-CoA and active formate.

ARGININE METABOLISM

The steps in citrulline synthesis have been further clarified. A full report on the isolation and characterization of N-acetylglutamic acid, the naturally occurring cofactor of carbamyl phosphate biosynthesis in liver and yeast appeared (278). Burnett & Cohen (279) purified the carbamylphosphate-ornithine transcarbamylase from beef liver approximately 100-fold. The equilibrium of the reaction strongly favors citrulline synthesis; the substrate specificity is high, and there is no apparent cofactor requirement. There was no indication of citrulline phosphate formation, although its existence as a highly unstable compound cannot be excluded. The phosphoro-

lysis of citrulline to ornithine plus ATP was reported by Smith (280) to occur in pleuropneumonia-like organisms. The effect of biotin deficiency on the ability of *Streptococcus lactis* to convert ornithine and carbamylphosphate to citrulline was the subject of a paper by Sund, Ravel & Shive (281). While this activity was greatly reduced in biotin-deficient cells, biotin alone could not restore activity. Glucose, phosphate, and amino acids were required in addition to biotin. Further, this restoration of activity was inhibited by purine, pyrimidine, and amino acid analogues. They concluded that there was a need for *de novo* enzyme synthesis in the presence of biotin.

Transamidination reactions in *Streptomyces griseus* were studied by Walker (282). An enzyme system in this organism catalyzed reversible arginine-to-ornithine and canavanine-to-ornithine reactions. An enzyme-amidine intermediate was indicated, since formamidine was trapped by the use of hydroxylamine. Formamidine disulfide inhibited this system.

Pearl & McDermott (283) have obtained evidence that the rate limiting step in urea synthesis is the conversion of citrulline to arginine. Arginine-glycine transamidinase, the enzyme giving rise to guanidinoacetic acid (the key intermediate in the synthesis of creatine), was previously described as being limited to the kidney, but has now been found in the pancreas (284). The same author described the presence of arginino-succinase in many tissues besides liver and kidney. A reproducible procedure for the purification and crystallization of arginase from beef liver was published by Bach & Killip (285).

Kalyankar *et al.* (286) described a new route for the breakdown of canavanine, viz. hydrolysis by a pseudomonad to hydroxyguanidine and homoserine. A scheme showing the seven known routes for the catabolism of canavanine was presented by them. Garcia & Couerbe (287) described the degradation of arginine via δ-guanido-α-ketovaleric acid to γ-guanidobutyric acid. The latter compound occurs in human urine together with δ-guanido-*n*-valeric acid (288).

Ravel *et al.* (289) studied the inhibitory effects of O-carbamylserine, S-carbamylcysteine, and azaserine on the growth of *S. lactis* and *Lactobacillus arabinosus*. In contrast to the competitive reversal of carbamylserine inhibition by glutamine, the inhibitions by S-carbamylcysteine and by azaserine were not competitively reversed. S-carbamylcysteine inhibited the conversion of ornithine to citrulline in cell-free extracts. This inhibition was not enough to account for the decreased growth, however, and it was found that cell-free extracts of cells grown in the presence of these inhibitors contained smaller amounts of the ornithine-citrulline enzyme.

Studies on arginine in tissue culture systems have been made by Thomas *et al.* (290) and by Morgan *et al.* (291). In a synthetic medium, arginine was essential. It could not be replaced by ornithine and was replaceable by citrulline only at high concentrations. Canavanine was toxic, and its toxicity was reversed by arginine but not by citrulline or ornithine.

Westall (292) described the presence of argininosuccinic acid in urine,

plasma, and cerebrospinal fluid associated with severe mental deficiency in a new familial disease.

POLYAMINES

The biosynthesis of spermidine [N-(3-aminopropyl)-1,4-butanediamine] occurs in *E. coli* according to the following pathway (293): ATP + L-methionine $\longrightarrow$ S-adenosylmethionine $\longrightarrow$ CO_2 + S-adenosyl(5')-3-methylmercaptopropylamine. The addition of putrescine then gives spermidine and, probably, thiomethyladenosine.

The degradation of di- and polyamines in *Hemophilus parainfluenzae* and in *Neisseria perflava* was studied by Weaver & Herbst (294, 295). Both of these organisms degrade spermine and spermidine to 1,3-propanediamine, while N-(4-aminobutyl)-1,4-butanediamine is converted to putrescine. The inducible polyamine oxidase of *N. perflava* degrades spermine or spermidine to 1,3-propanediamine with the formation of hydrogen peroxide and an aldehyde.

Razin *et al.* (296) demonstrated the formation of β-alanine from spermine and spermidine by *P. aeruginosa.*

Ames *et al.* (297) identified putrescine and spermidine as components of liver nuclei, *E. coli* cells, and the T4 phage. These compounds were identical with the A_1 and A_2 components of phage which enter *E. coli* together with DNA. The amount of the polyamines present in T4 was sufficient to neutralize one-third to one-half of the DNA phosphate.

DIAMINOPIMELIC ACID

It is known that N-succinyl-L-2,6-diaminopimelic acid is a precursor of diaminopimelic acid in *E. coli* and that aspartic acid contributes four of the seven carbons in diaminopimelic acid. Gilvarg *et al.* (298) demonstrated that the succinyl group is attached to the end of the diaminopimelic acid molecule which arises from aspartic acid.

The metabolism of diaminopimelic acid is closely related to the process of spore germination. On one count, it is a constituent of the "spore peptide," a material released from the spore coat during germination. On a second count, it may well be a precursor of pyridine-2,6-dicarboxylic acid, the calcium salt of which accounts for 10 to 15 per cent of the dry weight of the resting spore. Powell (299) found a parallel increase in diaminopimelic acid decarboxylase and vitamin B_6 content of *B. sphaericus* spores during germination. The low conversion of diaminopimelic acid to pyridine-2,6-dicarboxylic acid in germinating spores may be caused by permeation difficulties (300), and although these authors favor a C_4 plus C_3 condensation mechanism for the formation of pyridine-2,6-dicarboxylic acid, diaminopimelic acid is not excluded as an intermediate. It seems clear from this and other work (162, 301) that the pathway—tryptophan to 3-hydroxyanthranilic acid —for pyridine biosynthesis is not the only one operative in biological systems.

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METABOLISM OF STEROIDS[1,2]

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ADRENAL STEROIDS

Since knowledge concerning the adrenocortical steroids has been accumulating over many years, we have treated them as a group, singling out only aldosterone, which has been the subject of several reviews (56, 81, 82, 84, 97, 123, 148), for individual attention.

Aldosterone.—The origin of aldosterone is accepted as the adrenal cortex. Giroud and co-workers showed *in vitro* that the zona glomerulosa contained most of the aldosterone-secreting activity of the intact gland (97 p. 56); similar observations have been made with beef adrenals (2). Wettstein *et al.* (148) have shown that by aerobic incubation in suitable media, beef adrenal can be induced to synthesize many times the amounts produced by hog adrenals under similar conditions. Suggestive evidence of extra-adrenal production of sodium-retaining factor was reported by Girerd & Green (56) in urine of adrenalectomized hypertensive patients.

The precursors of this hormone have not been clearly defined; increases in aldosterone production by beef adrenal preparations were observed after addition of progesterone, 11β-hydroxyprogesterone, DOC, Compound A, or corticosterone (148, 114, 97, p. 56). Use of radioprogesterone permitted isolation of isotopically labeled aldosterone (20), while Travis & Farrell (133) isolated aldosterone having a specific activity similar to that of the progesterone-4-C^{14} used as substrate from beef adrenal incubates. They noted after corticosterone-4-C^{14} was used as substrate that the aldosterone isolated had only 54 per cent of the molar specific activity of the substrate, implying that the latter is not an obligatory intermediate. More recently, Seltzer *et al.* (122) presented evidence of increased aldosterone production in intact humans after injection of corticosterone. Since these conversions are less than 1 per cent, search for more efficient precursors and cofactors might be indicated.

Authors are generally agreed that the feedback mechanisms which regu-

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[1] The survey of the literature pertaining to this review was completed by November 1, 1958.

[2] In order to conserve space, we have used the following abbreviations: DHA for dehydroepi-(or iso)androsterone, DOC for cortexone (11-deoxycorticosterone), KS for ketosteroid, TH for tetrahydro derivatives. THA is the abbreviation for tetrahydro compound A; A, B, E and F refer to designations of compounds used by Kendall; S is the abbreviation used by Reichstein.

late glucocorticoid output are relatively ineffective in controlling secretion of aldosterone. *In vivo* and *in vitro* experiments have shown that purified growth hormone does not significantly increase aldosterone in blood, urine, or incubation mixtures (5, 116, 124, 143), while ACTH has been reported variously to cause minimal increase or decrease, or have no effect on aldosterone secretion (41, 42, 97, 123, 141, 150). In rat, dog, and man, hypophysectomy led to marked decreases in other corticosteroids, while aldosterone production was much less affected (83, 124). Administration of ACTH to normal men produced a fall in aldosterone and a simultaneous rise in hydrocortisone production (123).

The influence of nerve impulses to the adrenal was ruled out as the stimulus to aldosterone secretion by the transplantation experiments of Fleming & Farrell (45), who found no significant change in cortisol production but a doubling of aldosterone secretion by the transplant. In search of a humoral agent, they examined the role of the diencephalon (110). The influence of extracts of posterior pituitary was examined by Giroud *et al.* (97, p. 66). Their data suggest that neither oxytocin nor vasopressin is active but that an unknown substance in posterior pituitary extracts increases aldosterone production by beef adrenal slices with or without added DOC. Orti, Ralli *et al.* (104) have reported the presence of an aldosterone-stimulating substance in urine of rats deprived of salt.

Other factors which reportedly increase aldosterone output include the electrolyte intake, decreasing Na or increasing K intake (72, 82, 91), blood loss (43, 44), fluid volume changes (4, 97), trauma (144), surgery (52, 53, 144), anxiety (142), position and activity (96, 97), pregnancy (3, 76, 80, 145), eclampsia (3, 76, 145), and cases of primary (21) and secondary aldosteronism (28, 81, 100, 149).

Decreases in aldosterone output result from sodium loading or potassium restriction (82, 91), expansion of the extracellular space (47), and ablation of the adrenal cortex by disease (97), surgery, or suppression by chemical attack *(vide infra)*. In this connection it has been reported by Baulieu *et al.* (146) that decrease of elevated aldosteronuria to normal has been achieved in ascitic cirrhotics by cortisone therapy; similar results were obtained in experimental nephrosis by Das Gupta (23). A patient with symptoms including hypotension and hyponatremia and with no detectable aldosterone in her urine, even on sodium restricted diets, has been described as pure hypoaldosteronism by Skanse & Hökfelt (125).

Accurate determination of aldosterone in any biological sample is difficult, time consuming, and the significance of the result may be doubtful. Levels in daily urines have been summarized (100); the range of normal output varies with technique but is reported to be between 1 and 9 μg. per 24 hr. (30, 54, 65, 95, 100, 101, 103, 112, 150). Neher has recently presented an interesting appraisal of assay methods (99). In experiments with operated dogs, Davis *et al.* (24) showed that, after inferior vena caval constriction with resultant heart failure, six times as much aldosterone appears

in adrenal venous blood as in the control animals. Assay of the urines from these experiments yielded less than 1 per cent of the aldosterone which appeared in blood. Thus the factors regulating metabolism of the remaining 99 per cent of the aldosterone may be of prime importance.

An approach to the elucidation of this problem has been initiated by Ayres et al. (1), who have determined the biological half life of tritiated aldosterone in normal men to be 18 to 25 min. This datum, compared with the half lives of corticosterone, 1 hr., and of cortisol, 1.4 hr., is interpreted by the authors as indicating more rapid metabolism of aldosterone, possibly to an easily split glucuronide. In an extension of their work (97, p. 73), they were unable to account for the rapid disappearance of the free hormone as a glucuronide or as a conjugate labile to continuous extraction at pH 1, as is the case in urine. Approximately 60 per cent of the administered tritium was recovered from the first 2 days' urine, a large portion of the labeled compounds being hydrolyzed very slowly in the presence of β-glucuronidase. About 5 per cent of the metabolite was recovered as aldosterone and about 25 μg. of a metabolite which they suggest might be tetrahydroaldosterone. This compound has been tentatively identified by Ulick & Lieberman from human urine (138).

On the basis of their data, Ayres et al. (97, p. 94) state that the concentration of aldosterone in the peripheral blood of man is about .03 μg. per cent, that their subject on a normal salt intake secreted 190 μg. of aldosterone per day, and on a restricted sodium diet 780 μg. per day, of which 7.2 and 42 μg. respectively appeared in the urine.

Other factors making investigation of aldosterone more difficult are the limited supplies of the hormone resulting from expense and difficulties in preparation; presumptive evidence of a compound with chromatographic properties similar or identical to those of aldosterone, which on bioassay leads to enhanced sodium excretion (56, 75, 97, 145); the possibility, suggested by Eberlein & Bongiovanni (32), that for optimal aldosterone activity minimal amounts of hydrocortisone are necessary; the marked differences between "normal" urines collected from various geographic-socio-economic and dietary areas of the world (97, p. 25); and diurnal variations in aldosterone output (97).

A fascinating new chapter in the metabolism of steroids is rapidly being written. Various principles valid in other disciplines employing antagonists have been applied to this field: substances competing for a specific metabolic process, compounds with demonstrated toxicity or lethality for the adrenal cortex (15, 46), and structural antagonists effective both biochemically and pharmacologically.

Corte & Johnson demonstrated higher blood levels of 17-hydroxycorticoids after simultaneous administration of cortisol and N-acetyl-p-aminophenol. They suggest that competition for glucuronide conjugating enzymes delays metabolism of the corticoid (22).

A compound which has been shown to inhibit steroidogenesis in the per-

fused calf adrenal is amphenone B (1,2-*bis*-(*p*-aminophenyl)-2-methyl-1-propanone-2HCl) (115). Hertz *et al.* pointed out that amphenone depressed the adrenal response to ACTH in the hypophysectomized dog for periods up to 3 hr.; this depression was rapidly reversed in the normal dog (137). Application of this finding to humans with hyperadrenocorticism was rapid (51, 66, 67, 94). Amphenone given to a nephrotic not only decreased the urinary 17-hydroxycorticoids but also caused a precipitous fall in urinary aldosterone levels (84). Evidence has been obtained that the mechanism of action of amphenone is by inhibition of 11β-hydroxylase, with consequent decrease in plasma 11-hydroxylated corticosteroids and increased amounts of Substance S. That this occurs has been shown for a new amphenone analogue, 2-methyl-1,2-*bis*-(3-pyridyl)-1-propanone (71, 89, 90, 115). Unfortunately, the usefulness of amphenone is limited because of its toxicity, but it is hoped that newer derivatives will increase therapeutic effectiveness while decreasing toxicity.

Gaunt ends his excellent review (55) with the statement: "Discovery of some practical means by which aldosterone secretion may be inhibited would provide a welcome therapeutic tool." Amphenone or related compounds may well be this tool, but of exceeding interest are the two compounds reported by Kagawa *et al.* (73). These are reported to inhibit competitively the action of deoxycorticosterone and aldosterone in animals, leading to natriuresis. In his study of seven cardiacs with edema, Liddle found that 1.2 gm. per day of the "Spirolactone" induced consistent sodium losses. Interestingly enough, he reported that to be effective these compounds require the presence of DOC or aldosterone; natriuresis did not occur in an untreated Addisonian or in normal individuals until mineralocorticoids were elevated (87, 88). McCrory & Eberlein (93) observed a natriuretic effect of Spirolactone in four normal patients and one twelve-year-old male with hypokalemic alkalosis; they suggest that the antagonist alters H+ excretion in the renal tubule. Sturtevant showed that the parent Spirolactone possessed hypotensive effects in metacorticoid hypertensive rats while the 19-nor derivative, although a stronger natriuretic, was without pressor effects (127). Spirolactone has been reported to be effective in primary aldosteronism (119) and in mineralocorticoid excess caused by an adrenal cortical adenoma (84). If these results are confirmed, it would appear that a new era in the treatment of edematous states is at hand.

Corticoids from adrenal tissue.—Neher & Wettstein (102) isolated nine pregnane derivatives from adrenal tissue; five were new compounds, and none of the other four had been obtained previously from this tissue. After incubation of deoxycorticosterone with homogenates of adrenals 4-pregnene-6β,17α,21-triol-3,20-dione, 4-pregnene-6β,11β,21-triol-3,20-dione, 4 pregnene-11β,19,21-triol-3,20-dione, 4-pregnene-17α,20α,21-triol-3,11-dione, and 4-pregnene-19,21-diol-3,20-dione were isolated and identified. The last four and four other compounds (4-pregnene-17α,19,21-triol-3,20-dione, allopregnane-11β,17α,21-triol-3,20-dione, 4-pregnene-6β,21-diol-3,11,20-trione, and

4-pregnene-20,20,21-triol-3-one-18,20-acid lactone) were isolated from swine or bovine adrenals or both and fully characterized. Of particular interest are those compounds hydroxylated in the 6β and 19 positions and the 18,20 acid lactone.

In further studies with bovine adrenal homogenate, Eichhorn & Hechter (33) have found that deoxycorticosterone-21-C^{14} is not converted to cortisol but to an unidentified product which can be separated from cortisol by careful paper chromatography. Comparison of the amounts of products formed from 11β-hydroxyprogesterone, progesterone, and deoxycorticosterone indicates that the former is not a major intermediary in the biosynthesis of corticosterone and cortisol from progesterone [Eichhorn & Hechter (34)]. Incubation of progesterone-4-C^{14} and a cell-free preparation from hog adrenals gave radioactive 17-KS, and although 17α-hydroxyprogesterone was one of the principal products, some 16α-hydroxyprogesterone was detected [Rao & Heard (109)].

Eik-Nes & Brizzee (35) reported that plasma 17-hydroxycorticoids fell to very low values after hypophysectomy. In hypophysectomized dogs the cortisol production by the adrenal perfused *in situ* was very low; addition of ACTH to the perfusing fluid increased the secretion fourfold [Hilton *et al.* (68)]. The effect of ACTH on the rate of production of steroids by adrenal tissue *in vitro* is influenced to a marked degree by the presence of a TPNH-generating system [Haynes & Berthet (62); Koritz & Peron (77)].

Corticoids in blood.—Bush & Sandberg (17) have shown by elaborate characterization that the principal 17-hydroxycorticoid is cortisol and by partial characterization that corticosterone is also present in some specimens of human blood. By using a large volume of plasma, Peterson (106) has more completely identified corticosterone in human plasma.

That the difficulties besetting the determination of plasma corticoids have been recognized by many investigators is obvious from the procedures introduced to attain greater precision. Recently Bondy *et al.* (9, 10) and Peterson (106) have added trace amounts of cortisol-4-C^{14} and corticosterone-4-C^{14}, and Ayres and co-workers (1) have used corticosterone-16-H^3 (over 1 μc. per μg.), to compensate for losses in extraction and purification. Reports show that the normal values for cortisol are about 10 μg. and for corticosterone about 1 μg. per 100 ml. of plasma.

Weichselbaum, Margraf & King (147) have modified the conditions [Ercoli *et al.* (40); Umberger (140)] for the determination of biologically active steroids (Δ⁴-3-keto) in blood plasma. The mean values measured by this procedure—13.3 μg. per 100 ml.—and by the Porter-Silber reaction—12.9 μg. per 100 ml.—show close agreement, but considerable differences frequently occur in some individual specimens of blood. The authors propose that Δ⁴-3-keto compounds which do not react with the Porter-Silber reagent may be present in plasma.

In normal subjects, values for free 17-hydroxycorticoids were 12.5 μg. per cent and for conjugated 17-hydroxycorticoids after hydrolysis with β-

glucuronidase 10.4 µg. per cent [Brown *et al.* (14)]. After infusion of cortisol, the rise in conjugated steroids lagged behind the free steroids. With tracer amounts of cortisone-4-C^{14} and cortisol-4-C^{14} Hellman *et al.* (64) observed a rapid elimination of metabolites of these compounds in urine. No appreciable quantity appeared in bile or feces.

In the human the half life of cortisol is 1.4 hr., of corticosterone 1 hr., and of aldosterone 0.3 to 0.4 hours [Ayres *et al.* (1)]. Levin & Daughaday (85) have reported that the rate of disappearance of cortisol from plasma indicates a retarded rate of degradation in myxedema and an accelerated rate in hyperthyroidism. In an experiment of similar type, Brown *et al.* (13) reported that the fall of 17-hydroxycorticoids and rise in conjugated steroid of plasma were more rapid in hyper- than in hypothyroidism. In uremia and in liver disease the disappearance of infused cortisol was slower than for normal subjects [Englert *et al.* (38); Englert et al. (39)]. Conjugation was apparently impaired in liver disease and retention was observed in uremia. The rate of metabolism of cortisone by the liver in an *in vivo* perfusion experiment in dogs was 31.5 mg. per hr., of cortisol 29.6 mg. per hr. [Hechter *et al.* (63)]. In another experiment, the half life of cortisol in the dog was 52 ± 12 min., about half the value for the human [Kuipers *et al.* (78)]. Cortisol, given intravenously, remains in the blood of "stressed" dogs longer than in normal dogs [Eik-Nes & Samuels (37)].

Dorfman (27) has recently tabulated a comprehensive list of cortical steroids and their metabolites which have been isolated from tissues and urine. Since then, Lewis (86) has reported qualitative evidence for the presence of cortisone in peripheral blood; since cortisone has not been identified in blood from adrenal veins, he suggests that it may be formed peripherally. Suggestive evidence, the position on the paper chromatogram and sulfuric acid absorption curve, for the presence of THA in a pool of serum from children injected with ACTH has been given by Klein *et al.* (74). Compound A has been isolated from the urine of a patient receiving corticotropin [Touchstone, Bulaschenko & Dohan (131)], thereby indicating its presence in blood. Romani (112) subjected extracts of plasma to hydrolysis with enzymes from *Helix pomatia* and after partial purification subjected the steroid fraction to chromatography on paper. He reported evidence for the presence of Compounds F, B, E, and A, THA, THB, and *allo* THB. In a pool of 1860 ml. of plasma cortisol, corticosterone, THF, and THE were identified, but THA was not detected [Tamm, Beckmann & Voigt (128)]. The presence of corticosterone and its dihydro derivative at C-20 in mouse blood has been reported [Southcott *et al.* (126)]. Eberlein & Bongiovanni (31) reported the presence of Substance S and THS in the blood of a patient having congenital adrenal hyperplasia. Touchstone (130) has identified Substance S in four of five specimens of human adrenal venous blood.

Corticoids in urine.—*Allo* THF has been isolated from human urine and identified [Bush & Willoughby (18); Romanoff *et al.* (113)]. The mean

values for urines of six men were: *allo* THF, 1.3; THF, 1.3; THE, 3.1 mg. per 24 hr. *Allo* THE was not detected (113). It has been suggested that trauma leads to increased excretion of the metabolites, THE, THF, and *allo* THF [Gold *et al.* (58)].4-Pregnene-11β,17α,20α,21-tetrol-3-one has been isolated from human urine after the administration of cortisol [Peterson *et al.* (107)]; in a heart-lung preparation in a dog, a metabolite of cortisol was obtained which appeared to be the 20β-epimer [Travis & Sayers (134)]. The major metabolite (9.5 per cent) after administration of cortisone to an adrenalectomized man was 4-pregnene-17α,20β,21-triol-3,11-dione [Lombardo & Hudson (92)].

The presence of THA, *allo* THB, Compound B, and THB in urine has been reported [Romani (112)]; Dyrenfurth *et al.* (29) have also identified the last two compounds. In a urine pool, the excretion of THS amounted to 20 μg. per day. It was detected in urines from several people, but it was not invariably present [Touchstone *et al.* (132)]. THS, pregnane-3α,17α,20,21-tetrol, pregnane-3α,21-diol-20-one and pregnane-3α,17α,20α-triol have been isolated by Bongiovanni & Eberlein (11, 31) in crystalline form from the urine of a patient having hypertensive congenital adrenal hyperplasia. These observations suggested a deficiency of 11β-hydroxylase in the adrenal. In the urine of another case of congenital adrenal hyperplasia, Fukushima & Gallagher (48) found a relative lack of 21-OH compounds [see also (70)] but several with oxygen at C-11. In addition to androgens having oxygen at C-11, pregnane-3α,17α,20α-triol-11-one, pregnane-3α11β,17α,20α-tetrol, allopregnane-3α,17α,20α-triol, and allopregnane-3α,17α,20β-triol were also identified. Administration of 4-pregnene-11β,17α-diol-3,20-dione (21-deoxycortisol) to a normal man gave no metabolites indicating 21-hydroxylation [Rosselet, Jailer & Lieberman (117)]. The principal pregnane derivatives in the urine were pregnane-3α,11β,17α-triol-20-one, pregnane-3α,11β,17α,20α-tetrol, and pregnane-3α,17α,20α-triol-11-one, all of which are C-21 deoxy compounds. (It was shown that hot acid converts pregnane-3α,11β,17α-20-tetrol to $\Delta^{9,11}$-pregnen-3α-ol-20-one.) In another experiment with 21-deoxycortisol, Fukushima & Gallagher (47) demonstrated that the formation of the 20-ketone from 17,20-dihydroxysteroid by acid could be circumvented by hydrolysis with β-glucuronidase and stated that the use of hot acid "for cleavage of conjugates should be abandoned." Subsequently, Fukushima & Gallagher (49) have shown that the presence of 21-deoxysteroids in the urine of patients with congenital adrenal hyperplasia is not caused by removal of the C-21 hydroxyl by metabolic processes of these patients; it is an "inborn error of metabolism."

Hydroxylation at C-21 is effected by microsomes of beef adrenal glands and TPNH or a TPNH generating system. Progesterone, 11β-hydroxyprogesterone, 17α-hydroxyprogesterone and 11β,17α-dihydroxyprogesterone were hydroxylated at C-21 [Ryan & Engel (118)]. Hofmann (69) stated that hydroxylation of the side chain by rat adrenals to produce Porter-Silber chromogens was less effective for 11β-hydroxy- than for 11-deoxysteroids.

Bush (16) has given a stimulating discussion of the 11-oxygen function. During the metabolism of 11β-hydroxy-Δ^4-androstene-3,17-dione in man, no significant loss of the oxygen function at C-11 was observed [Bradlow & Gallagher (12)]. 11β-Hydroxylation is carried out by three distinct enzymes —two of which have been partially purified—an unidentified heat stable coenzyme from liver, placenta, or adrenal homogenates, molecular oxygen, and TPNH [Tomkins et al. (129)]. In two-stage adrenalectomy with the patient treated with corticotropin prior to the second operation, homogenates of the last removed adrenal were more active in 11β-hydroxylation and formed more corticosterone and cortisol from deoxycorticosterone and its 17-hydroxy derivative than homogenates from the adrenal obtained at the first operation [Grant et al. (59)]. These observations were correlated with the histological appearance of the adrenals. Administration of 2-methyl-1,2-bis-(3-pyridyl)-1-propanone to normal subjects caused the appearance of a steroid in plasma which seemed to be Substance S and the excretion in the urine of a compound presumptively identified as THS. The compound also interfered with 11β-hydroxylation in vitro [Liddle et al. (90)]. The amount of cortisol in the blood from the adrenal vein of a dog given this compound fell to a very low value, and Substance S appeared; corticosterone disappeared, and deoxycorticosterone was detected. Administration to a man having Cushing's syndrome was followed by excretion of THS and of reduced amounts of THE and THF [Jenkins et al. (71)].

Using O^{18}, Hayano et al. (61) have shown that hydroxylation uses molecular oxygen. In further work with tritium-labeled pregnane-3,20-dione, Hayano et al. (60) have concluded that hydroxylation in both the 11β and 11α positions proceeds by simple replacement of H by OH.

Further study has shown that the C-20-keto reductase of rat liver is associated with microsomes; a TPNH-generating system is required for its activity [Recknagel (111)]. Homogenates of rat kidneys in the presence of a TPNH generating system reduced the 20-keto group to the 20β-hydroxyl [DeCourcy (25)]. This reduction is also effected in vitro by beef pancreas [Nabors & Berliner (98)]. After the infusion of normal men with large amounts of corticosterone, 20α-dihydrocorticosterone was identified in the blood [Southcott et al. (126)].

Some of the metabolites of cortisol in man are 17-KS possessing an oxygen function at C-11. In his recent summary, Gallagher (50) pointed out that etiocholane derivatives are more abundant than compounds of the androstane series. Other investigators have come to the same conclusion [Sandberg et al. (120); Baulieu & Jayle (6)].

In the guinea pig, metabolites of cortisol found in the urine of control animals are cortisol, cortisone, 6β-hydroxycortisol, 2α-hydroxycortisol, and the epimeric 20-hydroxy reduction products of cortisol. Administration of ACTH caused two- to eightfold increase in the amounts of these corticoids [Peron & Dorfman (105)]. Eik-Nes et al. (36) have identified cortisol in extracts of bile of guinea pig. They suggest that this animal has a limited hepatic capacity to convert the 3-keto group to hydroxyl.

4-Pregnene-11β,17α,20β,21-tetrol-3-one was identified in the muscles of rats taken 5 min. after the intracardiac injection of cortisol-4-C^{14},[DeVenuto & Westphal (26)]. Homogenates of the livers of male rats degraded 50 per cent of the side chain of added cortisone, female rats only 5 per cent. Castration of males reduced the capacity of their livers [Troop (136)]. The rate of destruction of cortisol and its synthetic derivatives by slices of rat livers was less than that of cortisone [Schriefers *et al.* (121)]. Ulrich (139) reported that the principal metabolites of cortisol by rat livers *in vivo* are pregnane-3α,11β,17α,20β,21-pentol and allopregnane-3β,11β,17α,20β,21-pentol; 4-pregnene-11β,17α,20β,21-tetrol-3-one and allopregnane-3α,17α,20β,21-tetrol-11-one were also detected. The metabolites of cortisol incubated with mouse connective tissue were 4-pregnene-11β,17α,20,21-tetrol-3-one, cortisone, pregnane-11β,17α,21-triol-3,20-dione, 4-pregnene-11β,21-diol-3,20-dione and 4-androstene-11β-ol-3,17-dione [Berliner & Dougherty (7)].

Reduction products of cortisone, THE, and THF have been isolated in crystalline form from cattle bile [Glick (57)].

Corticoids in placenta.—From human placenta were isolated aldosterone 3 μg., cortisol 4 μg., cortisone 90 μg., 11-dehydrocorticosterone 45 μg., and 4-pregnene-17α,20β,21-triol-3,11-dione 20 μg. per kg. [Berliner et al. (8)]. Pincus (108) reported the total output of steroids by perfusion with beef blood of placentas removed by Caesarean section to be about 5 mg. during the first 4 hr. Troen (135) found the mean production of corticoids (Porter-Silber reaction) of human placenta perfused for 12 hr. to be 8.2 mg. During the first hour 0.13 mg. was formed and during the twelfth hour, 1.5 mg.

The belief that steroids are formed from cholesterol has received further support by (*a*) the demonstration that C-21 in cortisol which has been obtained by incubation of cholesterol-21-C^{14} with adrenal glands is radioactive [Kurath *et al.* (79)]; (*b*) the demonstration that C_{20} of pregnane-3α,17α,21-triol-20-one biosynthesized from $CH_3C^{14}O_2H$ is radioactive and that C-21 is not radioactive [Caspi *et al.* (19)].

GONADAL STEROIDS

ANDROGENS

Since the potency of known androgens is variable, depending on the nature of the assay, and since the identity of some of the compounds which produce virilization in certain conditions is unknown, this review will consider measurements of neutral 17-KS as being relevant to androgen metabolism. These measurements do not include 17-hydroxy-C-19 steroids which may be important to this subject.

Androgen production by the testes.—New data support earlier observations of the presence and production of androgen in the testis. Testosterone, androstenedione, or both in human testes [Anliker *et al.* (1)] and in spermatic vein blood of humans and dogs with or without the stimulatory effect of chorionic gonadotropin have been determined (20, 59, 91). The decrease in the level of testosterone in human spermatic vein blood with advancing age is in accord with the concurrent diminution of urinary androsterone

and etiocholanolone [Hollander & Hollander (59)]. Halkerston *et al.* (55) showed that the treatment of sexually retarded men with chorionic gonadotropin increased the excretion of these steroids. Brinck-Johnsen & Eik-Nes (20) isolated 0.275 mg. of testosterone from the blood collected from one canine testis under gonadotropin stimulation during 189 min. and calculated that 0.525 mg. was actually present. Since the level of androstenedione was also higher, the amount of androgen produced was quite remarkable. Jungck *et al.* (67) reported a case of sexual precocity caused by an interstitial-cell tumor of a testis in which the total urinary 17-KS fell rapidly from 25.7 mg. per 24 hr. to normal after removal of the enlarged testis. It has been reported that endogenous or exogenous estrogen caused a decrease in the excretion of androsterone and etiocholanolone [Halkerston *et al.* (56); Hermann *et al.* (58)].

Testicular tissue *in vitro* converts progesterone to testosterone [Lynn & Brown (92); Viscelli *et al.* (146)]. The administration of progesterone increased fecal androgen in a normal, but not in a castrated, ram [Raeside (113)].

Androgen production by ovaries.—Observations supporting previous evidence of the capacity of the ovary to elaborate androgens have been described. Zander (161) identified androstenedione and 17α-hydroxyprogesterone in extracts of human ovaries.

In transplantation and in parabiotic experiments in rats, biological evidence indicated secretion of androgens by luteinized ovaries [Johnson (66); Kullander (79)]. In clinical studies, administration of gonadotropin to women during the luteal phase [Netter *et al.* (101)] was accompanied by increase in 17-KS; this did not occur in ovariectomized women [Keller & Hauser (71)].

Evidence obtained from studies of virilizing ovarian tumors is more ambiguous because of the possibility of accompanying adrenal cortical pathology or inclusion of adrenal cortical tissue in the tumor. In cases of arrhenoblastoma several factors point to the production of androgen by the pathological ovaries: detection of testosterone and androstenedione but no corticosteroids or estrogen in the tumor tissue [Ruttner (114)]; small or no decrease in excretion of 17-KS after cortisone administration [Cohen *et al.* (27)]; elevated excretion of androsterone and etiocholanolone [Pesonen & Mikkonen (106)]; prompt fall in urinary 17-KS and relief of symptoms after removal of the tumor in each instance.

Opinion is divided regarding the source of androgens causing the hirsutism in Stein-Leventhal syndrome. An elevated urinary androsterone or etiocholanolone with normal or slightly elevated DHA and lack of a significant suppressing action by cortisone administration, indicating an abnormal ovarian androgen production, have been reported in some cases [Johnsen (65); Pesonen & Mikkonen (106)]. On the other hand, Perloff *et al.* (105) and Gallagher *et al.* (51) have concluded that the pattern of urinary 17-KS in the Stein-Leventhal syndrome is similar to that in "idio-

pathic" hirsutism in which an excessive production of adrenal 11-deoxy-steroids is indicated. In these cases the excretion of androsterone and etio-cholanolone was readily depressed by administration of corticosteroid; excretion of 11-oxysteroids was diminished to a lesser extent.

Androgen production by the adrenal cortex.—Bloch *et al.* (14) found that incubation of acetate-1-C^{14} with slices of adrenal gland from a young woman with adrenogenital syndrome resulted in the formation of radioactive androstenedione, DHA, and 11β-hydroxyandrostenedione. ACTH markedly stimulated the synthesis of C_{19} steroids, as was manifested by the accumulation of 11β-hydroxyandrostenedione. The isolation of DHA from an adreno-cortical tumor may be regarded as evidence for its origin in the adrenal [Plantin *et al.* (108)].

Wilson *et al.* (157) found 11β-hydroxyandrostenedione to be the major C_{19} steroid in the urine of female mice bearing on ACTH-producing pituitary tumor. Since 11β-hydroxylation is presumed to occur in the adrenal, it is of interest to recall that Savard *et al.* (125) reported that incubation of slices of testicular interstitial-cell tumor with testosterone-C^{14} produced 11β-hydroxytestosterone and 11β-hydroxyandrostenedione.

Less direct information has been obtained from *in vivo* studies. After the intravenous injection in a man of a mixture of cholesterol-C^{14} and cholesterol-H^3, labeled THE, THF, 11-ketoetiocholanolone, androsterone, and etiocholanolone were found in the urine [Werbin *et al.* (152)]. The administration of androstenedione mainly increased the excretion of etiocholanolone with no formation of 11-oxy-17-ketosteroids [Jaoudé *et al.* (64)]. Bulbrook *et al.* (25) reported that most women continued to excrete etiocholanolone after adrenalectomy. Androsterone was rarely found and DHA was present only in a case of incomplete adrenalectomy.

With the development of suitable analytical methods, there has been renewed interest in the study of the excretion of 17-KS, particularly by patients with adrenocortical disease. Various kinds of evidence have led Gallagher (49) to conclude that the human adrenal secretes two main groups of compounds: (*a*) those oxygenated at C-11 and which are most important to the organism, and (*b*) those which do not have such an oxygen function and are dispensable. The excretion of 11-oxy-17-KS reflects the metabolism of cortisol since no evidence was obtained for the removal of the C-11 oxygen function [Bradlow & Gallagher (17)], while the excretion of 11-deoxy-17-KS reflects the production of 11-deoxycorticoids. On this basis and from measurements of the individual 17-KS excreted by normal people and by patients with virilizing adrenal disease, both untreated and treated with ACTH, cortisone, or amphenone, Gallagher and co-workers (50, 51) have presented evidence for the independence of the two pathways of metabolism of the adrenal hormones.

Others have also studied the nature of the urinary steroid metabolites in adrenal pathology and have attempted to correlate biochemical and clinical findings (21, 64, 65, 97, 105). From the collected data, it is difficult to draw

firm conclusions regarding relationships between individual 17-KS or groups of 17-KS and the type of adrenocortical abnormalitiy or effect of treatment. The great variability of the results may possibly be attributed to differences in methods used and inadequate identification of the pathological condition involved. The possibility of aberrant gonadal tissue in the adrenal or vice versa [Piyaratn & Rosahn (107)] and of differences between the metastic lesion and primary neoplasm should not be ignored [Gallagher (49)]. Perhaps biochemical techniques may be more reliable than the conventional pathological methods in determining the type of tissue involved. In addition, careful fractionation of all the 17-KS is necessary if the results are to be meaningful in the solution of this problem.

Studies of urinary 17-KS in cases of feminizing adrenocortical neoplasms (13, 29, 135, 147, 158) and in Cushing's disease (5, 7, 77) serve to emphasize the complexity of the problem of abnormal adrenocortical function and the need for its elucidation.

Androgens in urine.—After the administration of testosterone-4-C^{14} to man, most of the metabolites were rapidly eliminated in urine and very little by way of the bile or feces [Hellman *et al.* (57); Sandberg & Slaunwhite (119)]. The urinary excretion of metabolites of administered 11β-hydroxy-4-androstene-3-17-dione was even more rapid [Bradlow & Gallagher (17); Sandberg & Slaunwhite (121)]. In this instance, the principal metabolites were androstane-3α,11β-diol-17-one and etiocholan-3α-ol-11,17-dione (17).

Fotherby and his co-workers (47, 48) have isolated from the urine of normal men 16α-hydroxydehydroepiandrosterone a hitherto undescribed steroid, 5-androstene-3β,16β,17β-triol not obtained from a natural source before and 5-androstene-3β,17β-diol not previously isolated from this source. Engel *et al.* (44) isolated two new steroids, 19-norandrosterone and 19-noretiocholan-3α-ol-17-one, from the urine of patients treated with nortestosterone.

The 17-KS are excreted largely, if not entirely, in the form of glucuronides or sulfates. A number of studies to determine the distribution of the conjugates of individual steroids has been reported [Chemama *et al.* (26); Crepy *et al.* (30, 31); Kellie & Wade (73); Weinmann *et al.* (150, 151); Wotiz *et al.* (160)].

Androgens in blood.—The range of normal values for both sexes obtained by Migeon *et al.* (99), which shows large variations, include most of the values found by other investigators (102, 140). For men, 48.2 ± 15 μg. of DHA and 25. ± 10 μg. of androsterone per 100 ml. were found. Values for women of the same age were slightly lower. Kellie & Smith (72) reported a wider range of values, with those for women lower than for men.

The presence of 11-oxy-17-KS in peripheral blood has been reported by Kellie & Smith (72); Savard (123); and Tamm *et al.* (141).

The 17-KS of plasma are conjugated mainly with sulfuric acid; these

sulfates are cleared from plasma much more slowly than glucuronides. DHA is cleared less rapidly than androsterone [Bongiovanni & Eberlein (15); Kellie & Smith (72)].

Measurement.—The recent progress in the determination of urinary estrogens has been admirably reviewed by Bauld & Greenway (6), who describe the methods of Brown and Bauld which permit the separation and estimation of very small amounts of estrogens. Although the methods are widely accepted, the problem of hydrolyzing the conjugates of these steroids has not been completely resolved. For example, Brown [personal communication to Bauld (6)] found the β-glucuronidase prepared from *Patella vulgata* yields estriol fractions which are more difficult to purify than those obtained after acid hydrolysis, and, therefore, he has adopted the use of the latter. This hydrolytic procedure has recently been found unsatisfactory by Smith & Smith (134). Bacterial β-glucuronidase had been reported (68) to yield fractions from human pregnancy urine which were less contaminated than those after acid hydrolysis, and, more recently, Smith & Blackham (133) found that a bacterial preparation was superior to a mammalian product for the hydrolysis of the conjugated estrogens in this medium. In view of reports of the superiority of the bacterial enzyme for hydrolyzing corticosteroid conjugates and of the mammalian β-glucuronidase for cleaving pregnanediol glucuronide (28, 61, 131), together with the importance of these enzymes to the isolation of new estrogens, comparative studies of these enzymes are desirable (22, 45, 78, 84, 88, 89, 94).

For the bioassay of estradiol-17β, Villee and associates have developed a sensitive, *in vitro* method based on the stimulation of what they presumed to be a DPN-linked isocitric dehydrogenase in human placenta. The reaction was measured by the reduction of the added DPN or by the production of α-ketoglutarate (52, 144). Hurlock & Talalay (62, 63) have also developed methods for the enzymatic microdeterminations of steroids by utilizing highly purified preparations of a 3α-hydroxy- and a 3β- and 17β-hydroxysteroid dehydrogenase obtained from cell-free extracts of *Pseudomonas testosteroni.* The assays depend on the quantitative enzymatic interconversion of hydroxy- and ketosteroids and the measurement of the changes in concentration of DPNH. With regard to estrogens, estradiol-17β and estrone respond to the enzyme possessing 17β-hydroxysteroid dehydrogenase activity.

That the reactions of the enzymatic methods mentioned above are related was demonstrated by Talalay & Williams-Ashman (139) and confirmed by Villee & Hagerman (145). The acceleration produced by estradiol-17β of the reduction of DPN by placental extracts in the presence of isocitrate requires a TPN-reducing system and catalytic amounts of TPN and can be accounted for in terms of a TPN-specific isocitric dehydrogenase and a suitable steroid-sensitive transhydrogenase. Other TPN-specific enzymes,

for example glucose-6-PO_4 dehydrogenase, can act as a generator for TPNH in this reaction. The reversible interconversion of estradiol-17β and estrone (85), as well as that of other 17β-hydroxy and 17-ketosteroids, indicates therefore a function as hydrogen carriers or coenzymes for pyridine nucleotide transhydrogenases.

It appears that different catalytic proteins are concerned with similar reversible oxidations of hydroxyl groups at different positions (138) of the steroid molecule. Studies of purification and characterization and structure specificity of the placental estradiol-17β dehydrogenase have been reported (60, 82).

A number of other studies on the determination of estrogens have been published recently (2, 23, 33, 35, 81, 96, 110, 149). A complicating, though very interesting factor, has recently been introduced by the Smiths (134), that considerable estrogenic activity in human pregnancy urine is present which can not be accounted for by estriol, estrone, and estradiol. At least six components, beside the three just mentioned and five of the recently isolated estrogens, contribute significantly to the estrogenic activity of estriol, estrone, and estradiol. Yields and characteristics of these components vary with the hydrolytic method used and with the period of gestation.

Studies with cholesterol and acetate.—A number of papers dealing with the biogenesis of estrogens have appeared since the reviews by Dorfman (39) and Engel (43) were published. Werbin *et al.* (153) found estrone-C^{14} in the urine of a pregnant woman after the administration of cholesterol-4-C^{14}. Hydrolysis was carried out with β-glucuronidase, but apparently no attempt was made to hydrolyze the steroid sulfates. The relatively high radioactivity of the estrone may indicate that it is not necessary to assume a pathway of estrogen synthesis independent of cholesterol via progesterone and androstendione.

Savard *et al.* (124) concluded from their studies on acetate-1-C^{14} incorporation into various sterols and steroid hormones in the pregnant mare that the incorporation into progesterone and estrone proceeds rapidly and in a parallel fashion. The specific activities of the allopregnanolone and of estrone in the daily samples were approximately the same. The rate of incorporation of acetate into cholesterol of whole blood was slow, apparently indicating a relatively minor role of the circulating body cholesterol in the synthesis of steroid hormones in the mare.

Equilin and equilenin, isolated from the same urines, had approximately the same specific activity but lower than that of estrone. In agreement with earlier work, this suggests that in the mare the ring B-unsaturated estrogens are derived from acetate by a route which differs from that of progesterone and estrone.

Further evidence to support the earlier observations of estrogen synthesis by testes was obtained by Rabinowitz (111), who found that acetate-2-C^{14} was incorporated into estradiol-17β by testicular homogenates of man, dog, and cat and that incubating mevalonic acid-C^{14} with homogenates of human

testicular tissue from cases of prostatic carcinoma yielded radioactive cholesterol and estradiol. The radioactivity of the cholesterol was greater than that of the estradiol (112). Less direct evidence was provided by the detection of small amounts of estradiol in extracts of human testes (1) and increased estrogen in the urine of patients with interstitial-cell tumor of the testes (42, 58). Laufer & Sulman (83) found large amounts of estrogenic activity in the tumor tissue and urine of a dog with a Leydig cell tumor.

Biosynthesis of estrogens from C_{19}-steroid.—Evidence has continued to accumulate to support the earlier reports of the formation of estrogens from androgens by mammalian tissue. Ryan (116) reported a 40 to 60 per cent conversion of androstenedione to estrone by preparations of human placental microsomes. TPNH or a TPNH-generating system was required. With testosterone, estrone again appeared to be the major product. However, if the microsomes were freed of steroid 17-ol-dehydrogenase, testosterone was converted only to estradiol and androstenedione only to estrone (115). Estriol was not detected in any of these studies, but Wotiz *et al.* (159) reported the conversion of testosterone-C^{14} to estriol by ovarian tissue. Ryan (117) has reported that in his system there is conversion of 5-androstene-3β,16α,17β-triol to estriol. It would be interesting to ascertain whether 5-androstene-3β,16β,17β-triol, recently isolated (47), is converted to 16-epiestriol.

A few new studies have been published on the increased excretion of estrogen after treating patients with androgens (8, 18). Engel *et al.* (44) observed that the increased excretion of estrone by a postmenopausal woman with breast cancer treated with 19-nortestosterone was approximately the same as after testosterone administration.

The recent work of Kushinsky (80) and Levy & Talalay (87) may provide an important tool for studying the perplexing problem of aromatization. Kushinsky showed that incubation of free 19-nortestosterone with *Corynebacterium simplex* resulted in a mixture of estrone, estradiol, and a small amount of 19-norandrostenedione. 19-Nortestosterone acetate-4-C^{14} was converted to estradiol-17β-monoacetate in 79 per cent yield by this microorganism. This observation is similar to that made somewhat earlier by Levy & Talalay (87), who found that *Pseudomonas testosteroni* converted 19-nortestosterone to estrone in 67 per cent yield. Smaller amounts of estradiol-17β were also produced. Some androstane- and 4-androstene- compounds were converted principally to 1,4-androstadiene-3,17-dione and to a lesser extent to 1,4-androstadien-17β-ol-3-one. Also 4-estrene-3,17-dione was converted to estrone. It is hoped that studies with these microbiological systems may reveal the pathway of aromatization and permit the isolation of new intermediates which can be studied in mammalian systems. In this connection, Kushinsky (80) has obtained evidence for the presence of a 1- or 2-hydroxylated intermediate which yielded estradiol-17β-4-C^{14} on treatment with $KHCO_3$.

Estrogen production by the adrenal cortex.—Only indirect evidence has

been added to previous findings which indicate that the adrenal cortex may contribute to the formation of estrogen. As in the past, most of the studies have been carried out on the urine or tissue of patients with neoplastic disease.

Snaith (135) reported that a girl with a feminizing adrenal tumor excreted increased amounts of estrogen and 17-KS. These were reduced to normal levels by removal of the tumor. The 17-hydroxycorticoids were within the normal range both before and after surgery, and pregnanediol was not detected in the urine. Similarly, elevated estrogen was found in the urine of adult males with feminizing adrenocortical carcinoma (13, 70, 147, 158).

The studies of West, Damast & Pearson (154, 155) suggest that estrogens or precursors are produced by the adrenal cortex and that endogenous ACTH may be involved in the control of this estrogen production. Injection of ACTH into a post-menopausal woman with metastatic adrenocortical carcinoma and Cushing's syndrome produced, respectively, twelve- and fivefold increases in the excretion of estrone and estriol. Estradiol was not detected in the control period, and only trace amounts were obtained after ACTH was administered. The presence of estriol in the urine of a castrated, adrenalectomized, and hypophysectomized patient having metastatic breast cancer has been reported (155). Of related interest is the report of McBride (98) that the low levels of urinary estrogens excreted by normal postmenopausal women may not be significantly altered by bilateral oöphorectomy and are about the same as those of normal adult men.

Salhanick & Berliner (118) isolated and characterized progesterone and identified a fraction as equilenin from a feminizing adrenal carcinoma. The equilenin was obtained in amounts almost as large as those of progesterone. With regard to a possible route of biosynthesis of equilenin, the report of Mueller & Rumney (100) is of interest. It was found that mouse liver microsomes, in the presence of TPNH and oxygen, hydroxylated estradiol-16-C^{14} to produce 6β-hydroxyestradiol and 6-ketoestrone. Although it is reasonable to assume that hydroxylation of Ring B precedes its unsaturation, it does not necessarily follow that equilin and equilenin are formed in this manner from other preformed estrogens.

Isolation of new urinary estrogen metabolites: (a) 2-methoxyestrone.— Kraychy & Gallagher (78) isolated 2-methoxyestrone from human urine after administration of estradiol-17β-16-C^{14}. This finding was confirmed by Engel, Baggett & Carter (45). The isolated compound accounted for about 8 per cent of the administered radioactivity. More recently, Loke & Marrian (88) isolated 2-methoxyestrone from human pregnancy urine. In all instances, enzymatic hydrolysis was employed in the isolation procedure.

(b) 18-Hydroxyestrone.—The isolation and identification of 18-hydroxyestrone from the urine of pregnant women by Loke *et al.* (89) is most interesting, but its significance in estrogen metabolism is still not clear.

(c) 16-Hydroxyestrogens.—The notable achievement by Marrian and

his colleagues of the isolation of 16-epiestriol (148) and 16α-hydroxyestrone (94) has served to focus attention on 16-hydroxyestrogens and to add to the general interest in steroids of this type (12). Finding that the human excretes a considerable portion of injected 16α-hydroxyestrone as estriol without affecting the excretion of estrone or estradiol, Brown & Marrian (24, 93) suggested that estrone may undergo 16-hydroxylation to form the 16α and 16β-epimers which may then be reduced to estriol and 16-epiestriol, respectively. The recent isolation and identification of 16β-hydroxyestrone from the urine of humans during pregnancy [Layne & Marrian (84)] and after intravenous injection of estradiol-17β-16-C^{14} [Brown et al. (22)] supports this hypothesis.

The work of Levitz et al. (86) indicates that 16-ketoestradiol is an intermediate in the conversion of estriol to 16-epiestriol. The administration of estriol-16-C^{14} in human subjects led to the excretion of radioactive 16-ketoestradiol and 16-epiestriol. 16-Ketoestradiol gave both estriol and epiestriol as metabolites. Furthermore, 16-ketoestradiol was isolated from human pregnancy urine [Layne & Marrian (84)] and 16-ketoestrone has been reported to be a metabolite of estrone [Slaunwhite & Sandberg (132)]. Estriol and 16-epiestriol have been identified as metabolic products of 16-ketoestrone [Stimmel (137)].

Recently, Breuer et al. (19) reported that the incubation of 16α-hydroxyestrone with slices of human liver yielded estriol and 17-epiestriol (3,16α, 17α-triol); under the same conditions 16-ketoestradiol-17β was reduced to estriol and 16-epiestriol (3,16β,17β-triol.). Quantitative studies indicated that the 17-keto group was preferentially reduced to a 17β-hydroxyl while the reduction of the 16-keto group favored the formation of a 16α-hydroxyl. Diczfalusy & Halla (36) detected epimeric estriol in placental extracts which they believed was 16-epiestriol but the 17-epimer was not ruled out.

The conversion of radioactive estradiol to estriol by human ovarian tissue in vitro has been reported by Dowben & Rabinowitz (40). Most in vivo studies have demonstrated the occurrence of estriol in the urine after injection of estrone or estradiol. More recently, Brown, Fishman & Gallagher (22) estimated that following the administration of estradiol-16-C^{14}, 16β-hydroxyestrone accounted for 3 per cent, 16-epiestriol 6 per cent, and estriol 30 per cent of the total urinary radioactivity.

The role of estriol in the fetal metabolism of estrogen in the human appears to be of particular significance, even though it is not understood. Diczfalusy and co-workers (37, 38) found that estriol, almost entirely in conjugated form, was the main estrogen of amniotic fluid (little if any estrone or estradiol being present) and that the estrogen excreted by newborn boys is also almost solely conjugated estriol. Furthermore, after the injection of estradiol-17β, virtually no estrone or estradiol was detected in the urine. Since the gastrointestinal tract of the newborn is sterile, any difference in metabolism of estrogen might, at least in part, be accounted for by the effect of microorganisms in the intestine of older people. Sandberg & Slaunwhite

(120) have reported that about 50 per cent of the radioactivity of administered estrone-C^{14} and estradiol-C^{14} is excreted in bile and then reabsorbed and excreted in urine.

New sources of estradiol-17α.—Estradiol-17α has been found in the urine of pregnant cattle [Velle (142) and pregnant goats [Klyne & Wright (76)], but estradiol-17β was not detected. Injection of estradiol-17α, estradiol-17β, or estrone in calves resulted in the excretion of estrone and estradiol-17α but not of the 17β-isomer [Velle (143)]. Axelrod & Werthessen (3) obtained evidence for the conversion of estrone to estradiol-17α by blood; and Breuer *et al.* (19) reported the formation of 17-epiestriol from 16α-hydroxyestrone *in vitro.*

PROGESTERONE

Parturition is caused by a decrease in progesterone level or a change in the estrogen-progesterone ratio [Csapo (32); Bengtsson (9); Schofield (126)]. Csapo has cited evidence that the progesterone produced in the placenta may exert its effect locally on the myometrium by diffusion. From their work on rabbits, Schofield and Bengtsson concluded that progesterone dominates the myometrium throughout pregnancy, with the exception of the first and last days. The reactivity of the myometrium is lowest while progesterone is dominant and highest when estrogen in dominant.

Shore (128) states that the ovaries may be removed from the mare, cow, ewe, and bitch at some time during pregnancy without interruption of the pregnancy. He isolated a product which appeared to be progesterone only from mare's placenta. Subsequently, he identified 4-pregnen-20β-ol-3-one in extracts of that tissue from the mare and from normal and ovariectomized pregnant ewes [Short (129, 130)]. The amount of progesterone in blood of pregnant cows is very small, 0.7 to 0.98 μg. per 100 ml.; it decreases markedly on the day the calf is born. Progesterone which is added to blood from steers diminishes and is replaced by 4-pregnen-20β-ol-3-one. On the eighth day of the estrous cycle Edgar & Ronaldson (41) found 1.8 μg. of progesterone in 1 ml. of the venous blood from the ovaries of ewes. Values during pregnancy were similar but gradually fell to 0.15 μg. per ml. a few days before parturition. This may be related to the failure of the ewe to abort if the ovaries are removed during a late stage of pregnancy.

Zander & von Münstermann (164) have continued studies [Zander & Simmer (165)] of progesterone in tissues. None was detected in the endometrium, and only 0.14 μg. per ml. in amniotic fluid. The amount in human placenta—33 μg. in the second and third months—rises to 711 μg. in the ninth and tenth months; these figures correspond to 4.15 μg. and 1.98 μg. per gm. of tissue. Alkaline hydrolysis was not used in this work, since it was found to give smaller values than direct solvent extraction.

Two metabolites of progesterone [Zander *et al.* (162)], 4-pregnen-20β-ol-3-one and 4--pregnen-20α-ol-3-one were isolated from placenta, ripe follicles, corpora lutea, and fat tissue. By the Hooker-Forbes assay, the former

is twice as active and the latter 2/5 as active as progesterone. These compounds are regarded as metabolites because Wiest (156) identified the 20α-ol as a metabolite of progesterone in eviscerated rats, and Zander et al. (162) also found it in the fat of postmenopausal women after the injection of progesterone. Gorski et al. (53) have identified the 20β-ol in bovine ovaries; they found 0.79 μg. of it and 4.33 μg. of progesterone per gram of ovarian tissue.

In a re-investigation of the excretion of pregnanediol after daily injections of progesterone for several days, the "priming" effect was not observed [Marrian et al. (95)]. Klopper & Michie (74) likewise failed to find the "priming" effect; in nonpregnant women they found an average recovery of progesterone as pregnanediol of 12.9 per cent, and in pregnant women the values ranged from 6.4 to 21.9 per cent. Pearlman (103) has utilized the progesterone-16-H^3, which he synthesized to ascertain the amount of progesterone converted to tagged urinary pregnanediol; 6, 15, and 14 per cent was converted by pregnant women; 14 and 27 per cent by oöphorectomized hysterectomized women.

The concentration of progesterone in blood of the adrenal vein is 10 to 100 times that in arterial blood of pregnant cows [Balfour et al. (4)]. Klopper et al. (75) have cited the following data as evidence that progesterone is secreted by the adrenal. Urinary pregnanediol in adult males 0.52 to 1.86, postmenopausal women 0.52 to 2.0 mg. per 24 hr.; after injection with ACTH, values were 1.8 to 4.7 mg. per 24 hrs. From 4 to 10 days after adrenalectomy the values ranged from 0.1 to 0.40 mg. per 24 hr. Bergstrand & Gemzell (10) reported the excretion of pregnanediol by boys to be 0.76 and girls 0.72 mg. per 24 hr. Values as high as 10 mg. per 24 hr. were observed in urines of patients with congenital adrenal hyperplasia. The presence of pregnane-3α,17α,20α-triol and pregnane-3α,17α,20α-triol-11-one in the urine of patients having adrenal hyperplasia has been reported [Finkelstein & Cox (46)]; Bongiovanni & Eberlein (16) state that values for the triol may be as high as 60 mg. per 24 hr. Schwartz et al. (127) have reported that a very small proportion of injected progesterone is converted to pregnanediol by patients having cirrhosis of the liver.

In in vitro studies 6-ketoprogesterone has been identified in the perfusate of human placenta with bovine blood to which progesterone had been added [Hagopian et al. (54)]. Pregnane-3α,6α-diol-20-one has been found in the urine of a pregnant woman [Davis & Plotz (34)]; the amount increased after ovariectomy and again after adrenalectomy. The incubation of progesterone, 17-hydroxyprogesterone and 11-deoxycortisol with adrenal slices or homogenates gave cortisol; the yield from 17-hydroxyprogesterone (50 per cent) was three times the yields from the other compounds [Lombardo et al. (90)]. Homogenate of adrenal from a patient with Cushing's syndrome was very effective in converting progesterone to deoxycorticosterone 23 per cent, corticosterone 25 per cent, cortisol 23 per cent; no 17-hydroxyprogesterone was detected [Berliner et al. (11)]. Four enzymes, all of which re-

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quire TPNH, are required for the conversion of progesterone to androgen by testicular tissue. Oxygen is required by two of the enzymes. 4-Pregnene-17α,20β-diol-3-one was one of the products formed [Lynn & Brown (92)]. Incubation of progesterone with homogenate of bovine ovaries gave 17α-hydroxyprogesterone and 4-androstene-3,17-dione [Solomon et al. (136)].

In experiments in which from 1 to 2 μc. of progesterone-4-C^{14} were injected intravenously [Sandberg & Slaunwhite (122)], 52 per cent of the C^{14} was excreted in the urine; in three patients having bile fistulas, 53 per cent of the C^{14} was excreted in urine and 30 per cent in bile [see also Davis & Plotz (34)]. Clearance of C^{14} in "free" steroids was very rapid: only 2 per cent of the total amount remained in plasma after 15 min. At this time, C^{14} in the form of steroid glucuronide represented about 18 per cent of the injected progesterone. Pearlman (104) has estimated the turnover time, the time required for complete replacement of progesterone in blood by hormone from the endocrine glands, to be about 3 min. These figures, together with the estimates of Pearlman (103) and Zander & von Münstermann (163), indicate that about 250 mg. are secreted per day by the endocrine glands during pregnancy. The low concentrations of progesterone in blood, 0.08 μg. per ml. [Zander & Simmer (165)], indicate the speed with which it is metabolized.

Davis & Plotz (34) have examined certain tissues for C^{14} after the injection of progesterone-4-C^{14}. They were cognizant that the values included metabolites of C^{14}. The peak in blood occurred about 24 hr. after injection. Twelve hours after injection, C^{14} was found in the female organs, but the largest amounts were in body fat, 17 to 33 per cent [see also Kaufmann (69)]. Small amounts were also found in the fetal placenta [Plotz & Davis (109)]. Administration of cholesterol-H^3 and acetate-1-C^{14} to the pregnant woman led to a marked amount of cholesterol-C^{14} but very little cholesterol-H^3 in the liver and adrenals of the fetus. Presumably, the former was synthesized in the fetal organs and the latter resulted from absorption from the circulation.

BILE ACIDS

Methods.—The chromatographic systems developed by the Swedish workers (3) for the separation of the bile acids have been adopted by a number of investigators, largely without modification; however, Mirvish (44) has reported improved recoveries with the reversed phase column procedures after slight acidification of the movable phase. The column procedures of Mosbach et al. (44a) was used in modified form by Matschiner et al. (42) in their studies of the metabolism of the bile acids. Other recently reported methods are a colorimetric procedure based on the formation of hydroxamic acid derivatives of the bile acids (52) and a paper chromatographic system for the separation of the bile acids (13). Bergström and co-workers have applied tritium labeling in their continued studies of the metabolism of the bile acids (6, 7).

Isolation and characterization.—Several reports have appeared which describe the identification of bile acids in the bile of various species and in other sources. Nakayama & Johnston (45) identified chenodeoxycholic as a second major constituent of opossum bile. Anderson *et al.* (2) have reported the presence of cholic, chenodeoxycholic and tetrahydroxynorsterocholanic acids in the bile of the King penguin. These authors also collated data on the bile acids of birds. Dugal & Laframboise (14) have detected cholic and deoxycholic acids in the bile of the codfish. Evidence of the presence of cholic, deoxycholic, and chenodeoxycholic acids in the blood of normal human subjects was reported by Carey (12).

New bile acids continue to be uncovered and characterized. Haslewood obtained hyocholic acid, a new acid, from pig bile (3). Haslewood (26), Ziegler (54, 55), and Hsia *et al.* (27) have contributed to its characterization as $3\alpha,6\alpha,7\alpha$-trihydroxycholanic acid. Two other acids, newly isolated from rat bile (42), were also found to be $3\alpha,6,7$-trihydroxycholanic acids (27, 28). The fourth member of this series of $3\alpha,6,7$-trihydroxycholanic acids has not been found as a naturally occurring substance but was isolated as a metabolic product from the urine of surgically jaundiced rats after the administration of hyodeoxycholic acid-24-C^{14} (43). Partial synthesis of all of these acids has been reported (27 to 31).

The Kolbe electrolytic synthesis has been used in the partial synthesis of two isomeric (at C_{25}) $3\alpha,7\alpha,12\alpha$-trihydroxycoprostanic acids (9). The synthetic acids were identical with two trihydroxycoprostanic acids (α and β) previously isolated.

Wiggins (53) reported the isolation of an unsaturated acid from chicken bile and has characterized the substance as 3-keto-4,6-choladienic acid. The acid may be an artifact from 7α-hydroxy-3-keto-4-cholenic acid (2).

Biosynthesis.—Attempts to describe the intermediary steps between cholesterol and bile acid have led to the synthesis and metabolic studies of several labeled compounds. Some of these studies have been reviewed previously (3). Harold *et al.* have examined the metabolism of epicholesterol-4-C^{14} (25), 4-cholestenone-4-C^{14} (23), and cholestanol-4-C^{14} (24) in the rat. Although acidic products were obtained in each case, the principal acids of rat bile, taurocholic and taurochenodeoxycholic, were not secreted in labeled form. The chromatographic patterns of the unknown acids from all three substrates, both as free and as conjugated acids, were identical, and these observations contributed to a proposed metabolic interrelationship between the sterols studied.

The Swedish workers have found that $3\alpha,7\alpha$-dihydroxycoprostane (6) and 7α-hydroxycholesterol (33) give rise to taurocholic and taurochenodeoxycholic acids in the rat, and they suggest that one of the primary steps in the degradation of cholesterol in the rat may be hydroxylation at carbon-7 (33).

The administration of labeled coprocholic acid ($3\alpha,7\alpha,12\alpha$-coprostanic acid) to rats gave taurocholic and taurocoprocholic acids (10). There was

an apparent competition between oxidative and conjugative metabolisms; taurocoprocholic acid was recovered unchanged if given to rats with cannulated bile ducts (38).

Clarification of the intermediary steps between cholesterol and bile acid may come from studies of the metabolic activities of various tissue preparations, but progress in this direction has thus far been difficult. Frederickson (20) found at least four unidentified acids as well as the accumulation of a neutral metabolite during incubation of cholesterol with a mouse liver preparation. The neutral metabolite was identified (21) as a mixture of approximately equal amounts of 25- and 26-hydroxycholesterol. A purified sample of each of these compounds, labeled by incubation of cholesterol-4-C^{14}, was administered to rats with cannulated bile ducts, resulting in a rapid biliary recovery of C^{14} (21). Most of the radioactivity was saponifiable but no radioactive cholic acid was present.

Bergström *et al.* (4) observed an effective conversion of tritiated 3α,7α, 12α-trihydroxycoprostanic acid to taurocholic acid in rat liver homogenates.

Species differences in the conjugation of bile acids *in vitro* have been reported by Bremer (8), who also observed the conjugation of cholic acid with hypotaurine (15). The enzymatic activation of cholic acid by liver microsomes (3) has been further studied by Siperstein & Murray (51) and by Elliott (16, 17, 18).

Metabolism.—Other reports on the metabolism of bile acids *in vivo* have appeared. The new acids obtained from rat bile and characterized as 3α,6,7-trihydroxycholanic acids were identified as metabolites of chenodeoxycholic acids in the rat (42). It was further observed by Mahowald *et al.* (40) that one of these acids (Acid I) becomes the principal bile acid in the urine of surgically jaundiced rats. Quantitative as well as qualitative differences in bile acid metabolism in the jaundiced rat were obtained with deoxycholic (41) and hyodeoxycholic acids-24-C^{14} (43).

Rudman & Kendall (49) observed no bile acids in normal human serum or urine but consistently found significant levels in serum and urine of patients with obstructive jaundice. The ratio of trihydroxy to dihydroxy acids was affected by the nature of the hepatic disorder. Rudman & Kendall (50) also studied the binding of bile acids with plasma protein fractions. Albumin exhibited the greatest activity in this regard.

Several recent reports re-emphasize the potential role of intestinal microorganisms in the over-all picture of bile acid metabolism. Bacterial metabolites of cholic acid in the rat have been identified by Norman & Sjövall (46), who also observed that these metabolites can be absorbed in the cecum and eventually contribute to the composition of the bile. Deoxycholic, 3α,12α-dihydroxy-7-ketocholanic, and 3α-hydroxy-12-ketocholanic acids were identified as arising from cholic acid by microbial action. Microbiological transformation of cholic to deoxycholic acid has also been observed in the rabbit (39) and human (35). The conversion of cholic acid-24-C^{14} to deoxycholic acid in the intact human was demonstrated by isolation of

radioactive deoxycholic acid from the feces and from intraduodenally collected bile samples (35). Abell *et al.* (1) believe that microbiological transformation of cholic to deoxycholic acid occurs in the dog, and Portman & Murphy (48) have mentioned their observation that deoxycholic acid is a metabolite of cholic acid in the intact rat.

Regulating factors.—Earlier reports of the effect of thyroid activity on the secretion of bile acid have been confirmed and extended by Eriksson (19), who measured the secretion of both taurocholic and taurochenodeoxy-cholic acid in hyperthyroid, hypothyroid, and normal rats for five days after cannulation of the bile duct. In hyperthyroid animals, secretion of tauro-cholic acid decreased markedly but that of taurochenodeoxycholic increased. In the hypothyroid animals secretion of both acids decreased. Abell *et al.* (1) have reported evidence to indicate that hypothyroid dogs have an impaired capacity to convert excess dietary cholesterol into bile acids.

Portman *et al.* (47) reported a significant increase in the secretion of bile in rats on a diet supplemented with cholesterol. The same workers concluded that sucrose and dextrose are inhibitors of bile acid secretion. In a more recent paper, Portman & Murphy (48) used cholic acid-24-C^{14} to determine the effect of diet on fecal excretion of cholic acid and its metabolites. The greatest excretion occurred on Purina chow although the dilution of synthetic diet with 20 per cent·celluflour gave a mean excretion approaching that observed with Purina chow. Their data indicated an interrelationship between dietary and bacterial effects on the turnover of bile acid in the rat.

Bergström & Danielsson (5) reported decreased hepatic synthesis of cholic acid after intraduodenal administration of taurochenodeoxycholate to rats with cannulated bile ducts. The administration of unsaturated fats has been reported to lead to increased secretion of bile acid in the rat (11) and in human subjects with bile fistulas (32).

Lindstedt & Norman (36) examined the rates of fecal excretion of C^{14} after the administration of several labeled bile acids to rats and calculated half lives of two to three days for each acid. This half life was found to be extended to 10 to 15 days in rats treated with intestinal chemotherapeutics (37). A similar result was obtained with germ-free rats (22). The bile acid pool in man was approximately 3.5 gm. and the half life of cholic acid 2.8 days (34).

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BIOCHEMISTRY OF CARCINOGENESIS[1,2]

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The literature on the biochemistry of cancer now increases so fast that
the yearly increment can not be reviewed adequately in the space allotted in
the *Annual Reviews*. Last year Skipper & Bennett (1) coped with this
problem by restricting their review largely to the biochemical literature on
fundamental aspects of cancer chemotherapy. Similarly, in this review we
have emphasized the biochemical aspects of experimental carcinogenesis.
This subject last received consideration in these *Reviews* by Heidelberger
(2) in 1956. To bridge this gap we have included the more important de-
velopments reported since that time; the major emphasis, however, has been
on the literature from January 1957 to September 1958. Cancer research is
conducted by workers in many disciplines. Many of the references cited
below are not strictly biochemical but are important to a balanced view of
the phenomena collectively called cancer.

Among the more general reviews that have appeared, the penetrating
discussions by Burnet (3) and Huxley (4) on the biology of cancer, an
excellent symposium published in the *Journal of Chronic Diseases* (5), and
the extensive series of reviews under the editorship of Raven (6) deserve
special note. The more specialized reviews will be mentioned later. Limita-
tions in space have made it necessary to refer frequently to reviews rather
than to the original literature.

The discovery of new carcinogens continues but the mechanisms by
which cancer cells arise remain obscure and the subject of much speculation.
Research on this fundamental question has received particular impetus in
the past few years from the demonstrations that viruslike agents can be
recovered from certain leukemias and parotid gland tumors of mice (7 to
12). However, the demonstration that serial inoculations of cell-free prepa-
rations of those neoplasms will transmit information leading to the forma-
tion of these cell types in other animals does not necessarily indicate how
the primary tumors arose. Discussions of these problems in their present
state contain many semantic traps, not the least of which is the term "cause."

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[1] The excellent assistance of Mr. Victor Triolo in surveying the literature is
gratefully acknowledged.

[2] The following abbreviations are used in the text: AAF for 2-acetylamino-
fluorene; AF for 2-aminofluorene; BP for 3,4-benzpyrene; DAB for 4-dimethyl-
aminoazobenzene; DBA for 1,2,5,6-dibenzanthracene; DMBA for 9,10-dimethyl-
1,2-benzanthracene; DPN for diphosphopyridine nucleotide; DPNH for the reduced
diphosphopyridine nucleotide; MC for 3-methylcholanthrene. TPNH for the re-
duced triphosphopyridine nucleotide.

Far too few data are available to support any contention that the majority of primary cancers are "caused" by viruses.

Three general classes of carcinogens are recognized—radiations, non-viral inorganic and organic chemicals (usually of small molecular weight), and viruses and viruslike agents. The yield of tumors or the sites of tumor formation following the application of many of these carcinogens can be variously affected by the hormonal [Shimkin (13)] and nutritional [Tannenbaum & Silverstone (14), Tannenbaum (15)] status of the host or by the simultaneous or subsequent administration of inhibiting or cocarcinogenic substances (16). Collections of review articles citing the advances in many of these areas are to be found in the *British Medical Bulletin* issue on the "Causation of Cancer" (17) the volumes edited by Raven (6), the symposium in the *Journal of Chronic Diseases* (5), and the proceedings of the Second Canadian Cancer Conference (18). In a supplement to the original work by Hartwell (19), Shubik & Hartwell (20) have tabulated the literature from 1948 to 1953 on the carcinogenic activity of chemical compounds. These compilations are entitled *Survey of Compounds Which Have Been Tested for Carcinogenic Activity,* and they record all published tests of chemicals which lasted longer than 30 days. It is evident that many of the compounds listed were not tested adequately for carcinogenic activity.

CARCINOGENIC RADIATIONS

Increasing evidence indicates that both x-radiation and nuclear radiations can induce acute leukemia and chronic myeloid leukemia, but not chronic lymphatic leukemia, in man (21, 22, 23). The nature of the dose-response curve has received considerable attention. Court-Brown (21, 22) and Lewis (23) both suggested that it may be a nonthreshold effect. On the other hand, Kimball (24), Brues (25), Finkel (26), and Mole (27) have emphasized the statistical problems involved in evaluating the data and, for the most part, these investigators feel that the response is a nonlinear function of the dose and that there is a dose below which leukemia is not induced. Finkel (26) has shown that this is the case for the induction of leukemia and bone tumors in mice by Sr^{90}. Owen *et al.* (28) showed that six to eight week old rabbits were very susceptible to the induction of osteosarcomas from Sr^{90} while two-day-old and one-year-old rabbits were much less susceptible. Carcinogenesis by radioactive chemicals has been reviewed by Furth & Tullis (29).

The interesting results of Kaplan and his associates (30, 31, 32) that lymphomas may arise in C57BL thymi transplanted into previously thymectomized and x-radiated F_1 hybrid mice have been confirmed and extended by Law & Potter (33, 34). Whereas Kaplan *et al.* (32) by genetic tests found that all of the lymphomas which they tested arose from the transplanted thymic cells, Law & Potter by similar tests found that at least as many tumors arose from the host lymphoid cells as from the implanted, nonirradiated cells. One difference between the two series of experiments

was the longer time (up to 28 days) which elapsed in Law's experiments between irradiation and thymus implantation; in Kaplan's series the thymi were transplanted one hour after irradiation. Perhaps these results could be explained on the basis that x-radiation is an inciting agent for the endogenous production of a carcinogen and that immediately after irradiation the transplanted cells are more susceptible than the damaged cells of the host. The lipide peroxides which Horgan *et al.* (35) have found in the tissues of irradiated animals and which are toxic on injection into mice might be considered as a prototype of this kind of endogenously formed carcinogen. This type of mechanism could precede the effects of normal endogenous growth stimuli which Kaplan (36) has considered to be the real carcinogenic agents in this seemingly indirect carcinogenic effect of x-rays.

The known effects of radiations on cells have been reviewed by Upton (37), who has also summarized the suggestions on the ways in which radiations might prove carcinogenic. These are (a) by initiation of a multistage process which predisposes the cells to further alterations by growth-promoting forces in their environment, (b) by mutagenesis, or (c) through activation of a provirus or formation of a transforming agent within the cells. Much of the recent experimental literature on the induction of skin tumors in rats has been reviewed by Glücksmann (38), and the x-radiation- and I^{131}-induced thyroid neoplasms have been considered briefly by Doniach (39). X-radiation and exposure to fast neutrons elicited hyperplastic lesions and occasional adenocarcinomas of the glandular stomach (40) and benign and malignant mammary tumors (41, 42) in rats. The skin of mice receiving 8-methoxypsoralen in the diet was partially protected from the carcinogenic action of ultraviolet irradiation; intraperitoneal injection of the same compound just prior to irradiation appeared to accelerate tumor production (43).

CARCINOGENIC CHEMICALS

Polycyclic hydrocarbons.—Lacassagne, Buu-Hoi, Daudel & Zajdela (44) have presented a tabular summary of their data on the carcinogenic activity of 78 substituted angular benzacridine derivatives for mouse skin and subcutaneous tissue. They concluded that in this series the probability of finding a carcinogenic compound increases with the charge in the K region (high π electron density), but there are exceptions to this generalization and the activities for skin and subcutaneous tissue are not always equal. In testing a number of thioesters of carcinogenic hydrocarbons Waravdekar & Ranadive (45) found two which are very active in inducing fibrosarcomas in rats, although they have no obvious K region.

3-Methylcholanthrene, 3,4-benzpyrene, and 1,2,5,6-dibenzanthracene act similarly to 9,10-dimethyl-1,2-benzanthracene in initiating skin tumorigenesis when administered orally to mice (46). Marchant (47) found 40 to 70 per cent incidence of ovarian tumors in IF mice given repeated topical applications of DMBA; other strains were less responsive. Standish (48)

studied the pathogenesis of tumors induced in the submaxillary glands of rats by MC and DMBA, while Shubik & Della Porta (49) studied mice given repeated large doses of the hydrocarbons. Administered cutaneously, BP and MC induced skin tumors within 4 to 10 weeks; administered intra-peritoneally, these compounds and DMBA caused lesions of the hemato-poietic system similar to those induced by large doses of ionizing radiations or by nitrogen mustard.

The positive correlation reported by Heidelberger & Moldenhauer (50) between the levels of protein-bound derivatives of a series of C^{14}-labeled polycyclic hydrocarbons in mouse skin and their carcinogenic activities at this site has been extended by Oliverio & Heidelberger (51), who compared a number of hydroxy, methoxy, and acetoxy derivatives of DBA. Carruthers and his associates (52), who used fluorimetric assays, reported that appre-ciable levels of BP, MC, and DMBA (all strongly carcinogenic) were bound to mouse skin protein. Hadler *et al.* (53, 54) have studied the binding to skin protein of several C^{14}-labeled hydrocarbons related to DMBA. The latter authors, like Woodhouse (55), who used fluorimetry, are not con-vinced of the correlation between the extent of the formation of protein-bound derivatives of hydrocarbons and their carcinogenic activities for mouse skin. Hadler's results, however, are complicated by the high levels of radioactivity associated with the protein (presumably through nonmetabolic reactions) at very early times after administration of the hydrocarbons. Until the experimental procedures used by these investigators are altered to avoid this problem [see Gutmann & Peters (56)], interpretation of their data will be difficult. Eisner & McCarter (57) have reported the presence of a "loosely bound benzpyrene" in mouse skin. While certain noncarcinogenic or weakly carcinogenic hydrocarbons are bound at high levels to mouse skin proteins (50, 51), it appears significant that no carcinogenic hydrocarbon has yet been studied which does not yield appreciable levels of protein-bound derivatives on application to the skin. Thus, protein-binding can be con-sidered a necessary, but not sufficient, step toward tumor induction. For quantitative comparisons between hydrocarbons, fluorimetry is probably inferior to the use of C^{14}-labeled compounds. With fluorimetry the assump-tion must be made that the ratio of the fluorescence of the bound hydro-carbon to that of the compound administered is constant for all of the com-pounds under study. On the other hand, in using radioactive compounds it must be assumed that no C^{14} is bound to the protein except that which is still in a relatively intact ring structure. From the available data (58) this appears to be a valid assumption.

A new metabolite of BP, 5-hydroxy-3,4-benzpyrene, has been isolated from rat feces [Pihar & Spaleny (59)]. The BP metabolite originally designated X_1 by Weigert and Mottram is considered by Harper (60) to be a glucuronide of F_1. The latter metabolite has not been identified, but both Harper (60) and Conney, Miller & Miller (61) concluded that it is a mono-hydroxy compound different from 5-, 8-, or 10-hydroxy-3,4-benzpyrene. The

latter authors showed that liver microsomes from rats previously treated with BP rapidly oxidize the hydrocarbon to 8- and 10-hydroxy-3,4-benzpyrene and to F_1 via a TPNH- and oxygen-dependent reaction. Calcutt (62, 63) has continued his qualitative studies on the intracellular distribution of BP and other hydrocarbons. 3-Hydroxypyrene and its conjugates were identified as the major excretion products of pyrene; this metabolite is analogous to 8-hydroxy-3,4-benzpyrene and presumably arose through an intermediate formed in the liver [Harper (64, 65)]. Pihar (66) reported the co-oxidation of BP to hydroxy and quinone derivatives in the presence of cytochrome oxidase, cytochrome-*c*, and reducing agents. Two metabolites of DBA excreted by rabbits after administration of this hydrocarbon have been characterized by LaBudde & Heidelberger (67) as the 2'-hydroxy- and 2',6'-dihydroxy derivatives.

Diverse biochemical effects of the hydrocarbons have been observed. *In vivo* administration to rats of BP, MC, or certain other hydrocarbons rapidly causes greatly increased activities of several hepatic microsomal enzyme systems which metabolize the aminoazo dyes, BP, and 2-acetylaminofluorene [Miller, Miller, and associates (61, 68, 69)]. Induced enzyme synthesis is thought to occur in these instances. Repeated administration of MC to adolescent rats depressed certain hypophyseal functions [Huggins & Pollice (70)]. MC inhibited the growth of fibroblasts but not the epithelial cells of mouse prostates grown *in vitro;* after 4 to 10 days the treated epithelial cells divided more rapidly than those of the control cultures, but their DNA synthesis was not equally stimulated [Lasnitski & Pelc (71)]. When administered to partially hepatectomized rats, MC, DBA, DMBA, or BP stimulated liver regeneration [Gershbein (72)].

Tobacco tars and atmospheric pollutants.—The carcinogenicity and chemical nature of the tars from cigarettes have received much consideration as a consequence of the statistical studies implicating cigarette smoking as an etiological factor in at least some types of human lung cancer [reviewed in (73, 74)]. The consensus is that large repeated doses of the tars obtained by condensation of the smoke from cigarettes will induce skin tumors in mice [75 to 80] and rabbits (81); the activity must, however, be classified as weak. Of significance also is the observation of Gellhorn (82) that these tars have cocarcinogenic activity when administered after the application of BP. These tars were much less carcinogenic than BP or MC when introduced directly into the lungs of rats (83). A number of polycyclic aromatic hydrocarbons, both carcinogenic and noncarcinogenic, have been detected in condensates of tobacco smoke, but the amounts of the known carcinogens may be too small to account for the activity of the tars on mouse skin (77, 84 to 88). The possible significance of the numerous other compounds of many chemical types, as either initiators or cocarcinogens, should not be ignored (84, 89 to 92). Polycyclic aromatic hydrocarbons have also been identified in cigar smoke and in snuff (93, 94).

The importance of atmospheric pollutants derived from industrial plants,

vehicular exhausts, etc. as etiological factors in human lung cancer has been emphasized in reviews by Kotin (95) and by Kennaway & Lindsey (84), and further statistical evidence has been presented by Mills & Porter (96). A comparison of the hydrocarbon composition of atmospheric and exhaust soots was made by Lyons & Johnston (97). Falk *et al.* (98) showed that, although BP is contained in atmospheric soot, it was not present in soot particles from human lungs. BP and other hydrocarbons are apparently eluted from the soot *in vivo;* this may occur through solubilization by blood plasma proteins [Falk *et al.* (99)]. Kotin (95, 100) has emphasized that the carcinogenicity of atmospheric products cannot be attributed solely to its content of polycyclic hydrocarbons; oxidized products of aliphatic hydrocarbons are also implicated.

The literature on the carcinogenicity of mineral oil fractions and their contents of polycyclic hydrocarbons has been reviewed recently by Cook *et al.* (101). Several polycyclic aromatic hydrocarbons have been detected in oysters from oil-polluted waters and in coffee soots (102, 103), and 3,4,9,10-dibenzpyrene has been isolated from coal tar (104). Hueper & Cahnmann (105) have demonstrated the presence of other carcinogens in addition to BP in shale oil, and Sugiura, Smith & Sunderland (106) demonstrated that a high-boiling catalytically cracked oil was carcinogenic for rhesus monkeys. The carcinogenicity of creosote oils for mouse skin and lung has been demonstrated (107 to 110); the polycyclic hydrocarbons which have been identified in creosote oil do not appear to account for its activity.

Aminoazo dyes.—A study of fluoro-substituted derivatives of 4-dimethylaminoazobenzene by Miller *et al.* (111) pointed to the importance of a free 2-position for hepatocarcinogenic activity in this series; of the 13 dyes studied, all except the three substituted in both of the equivalent 2- and 6-positions had strong activity. The sarcomagenic activity of 4-dimethylaminophenylazo-1-naphthalene for rats was shown by Mulay & O'Gara (112); this compound is also an hepatocarcinogen for rats (113).

Liver tumor induction by DAB or its 3'-methyl derivative was inhibited by intravenous injections of Thorotrast or colloidal iron oxide (114), splenectomy (115), oral administration of certain benzimidazole derivatives (116), or feeding of sodium glucuronate or glucuronolactone (117). Ethionine fed simultaneously with 3'-methyl-4-dimethylaminoazobenzene had no effect on, or slightly inhibited, the induction of liver tumors (118), while rats fed ethionine for several weeks and then DAB developed more liver tumors than the controls fed either compound for the same periods (119). In the same manner as MC (120), BP, DBA, or 1,2-benzanthracene administered at low levels simultaneously with 3'-methyl-4-dimethylaminoazobenzene greatly inhibited the induction of liver tumors; pyrene was without effect [Miller *et al.* (121)]. Administration of MC similarly inhibited the induction of tumors by other aminoazo dyes and by 2-acetylaminofluorene (121, 122); chrysene was without effect on DAB carcinogenesis (122). The livers of rats fed the protective hydrocarbons have a greater capacity to

metabolize the dyes to noncarcinogenic compounds than the livers of rats fed dye alone (121). Baba (123) has confirmed the previously reported (124) inhibition of azo dye carcinogenesis by p-hydroxypropiophenone, but his histological studies do not suggest that the inhibition is related to a lack of pituitary function. Eversole (125) showed that adrenalectomized rats treated with deoxycorticosterone trimethylacetate were markedly protected from the carcinogenic effect of 3'-methyl-4-dimethylaminoazobenzene. The treatment with deoxycorticosterone was apparently necessary to inhibit the growth of accessory adrenal tissue in these rats.

The over-all distribution of C^{14} in the tissues and excreta of rats fed 3'-methyl-C^{14}-4-dimethylaminoazobenzene has been determined by Salzberg (126). The enzymes in the rat liver which oxidatively N-demethylate the aminoazo dyes are located in the microsomes and require oxygen and TPNH [Conney, Brown, Miller & Miller (127); Hultin (128)]. The activities of both the N-demethylase and azo linkage reductase systems are increased severalfold (apparently by the synthesis of more enzyme) after treatment of young rats with MC or certain other hydrocarbons [Conney, Miller & Miller (68)]. The destruction of DAB (presumably by reduction of the azo linkage) is inhibited *in vitro* by certain benzimidazoles and other compounds; the inhibition is partially overcome by flavin adeninedinucleotide [Clayton (129)].

Earlier studies by the Millers (130) pointed to the importance of protein-bound derivatives of the aminoazo dyes in the liver in the induction of hepatic tumors by these carcinogens; these derivatives have received further study. Rats given single 30 to 50 mg. doses of DAB or related dyes form protein-bound derivatives in the liver within a short time; at 24 hr. the levels are comparable to those obtained with continuous feeding of the dye in the diet (131 to 134). The bound derivatives formed *in vivo* under either regime are similar in most respects [Gelboin, Miller & Miller (131)], although Terayama *et al.* (134) suggested that the polar dyes released from the protein may differ in the state of methylation of the amino group. On the other hand the protein-bound derivatives formed on incubation of rat liver preparations with primary or secondary aminoazo dyes *in vitro* differ in several respects from the bound derivatives formed *in vivo* [Hultin (128); Gelboin, Miller & Miller (135, 136)]. When a microsome-TPNH system was used, the formation of nondye metabolites, which also unite with the liver protein, seriously interfered with the use of C^{14}-labeled dyes as substrates for this reaction (135).

Terayama and his associates (134, 137, 138) and Miller & Miller (139) have investigated the nature of the dye bound to liver protein *in vivo*. These studies are complicated by the small amounts of dye available and by the finding of several dye-containing fractions in either alkaline or proteolytic digests. Chromatography of extracts of proteolytic digests appears to yield dye-containing peptides, although rigid proof that the dyes are attached to the peptides has not been presented. The site of attachment of the protein to

the dye is also not clear. The lack of bound-dye formation from 2,6-difluoro-4-dimethylaminoazobenzene has been cited as favoring the 2-position (111). Terayama *et al.* (138) recently suggested the 3-position, but no evidence for the postulated structure was presented. The latter group (140) have also reported preliminary investigations on the protein-bound derivatives of *o*-aminoazotoluene. Sorof *et al.* (141) have resolved the "h" proteins, which contain most, if not all, of the bound dye in the supernatant fraction of rat liver, into six subcomponents by electrophoresis. The amount of one fraction—designated "slow-h_2"—increased when a carcinogenic aminoazo dye or 2-acetylaminofluorene was fed but not when the noncarcinogenic dye 2-methyl-4-dimethylaminoazobenzene was given to rats. The level of protein-bound dye in the livers of hypophysectomized rats, which are very resistant to liver tumor induction by the dyes, was lower than that of control rats fed *ad libitum* but not strikingly different from that of rats restricted to the same food consumption [Ward & Spain (142)]. The lower tumor incidences in rats fed MC, BP, or DBA with 3'-methyl-4-dimethylaminoazobenzene were paralleled by lower levels of protein-bound dye [Miller *et al.* (121)].

Hughes and his associates (143, 144) have reproduced and extended the striking observation of Weiler (145) that, in the livers of rats fed DAB, there are groups of cells which have lost the affinity of normal liver cells toward rabbit globulin-fluorescein complexes, although they are morphologically indistinguishable from near-by cells which retain this property. The induced tumors also lack this property (145, 146). Hughes (144) found a correlation between the rate of appearance of these nonstaining groups of liver cells and the hepatocarcinogenicity of the dye fed. Contrary to the report by Weiler, the staining reaction cannot depend upon an organ-specific antigen-antibody reaction since globulin from nonimmunized rabbits acted similarly to that from rabbits immunized with rat liver preparations (143); however, the results do point to a protein difference between the staining and nonstaining cells.

Investigations of the comparative composition of normal liver, liver from dye-fed animals, and the induced liver tumors have continued in attempts to gain insight into the primary metabolic alterations leading to malignant growth. Petermann *et al.* (147) demonstrated a reduction in the concentration of microsomes and increase in the amount of one of the soluble nucleoprotein fractions in induced liver tumors. Allard, de Lamirande & Cantero (148), Deckers-Passau, Maisin & de Duve (149), and Kishi (150) studied a variety of enzymes including RNAase, various phosphatases, esterases, desamidases, and cathepsin, while Reid & Lewin (151) considered purine-metabolizing systems. The incorporation of P^{32} and glycine-2-C^{14} into various nuclear components was investigated by Grant & Rees (152). Weinhouse and his associates (153, 154) examined acetate metabolism and the levels of DPN and DPNH. In general these studies and those reported in earlier years show that the induced liver tumors differ greatly from normal liver and generally contain less of the enzymes under study. The

alterations in the livers of dye-fed rats have usually been less striking, and it is difficult to determine whether the altered composition of the tumors is of prime importance or is a secondary change. In this connection it is pertinent that Stewart & Snell (155) recently reviewed the histopathogenesis of induced liver tumors of the rat and concluded that the hepatic parenchymal cell is probably the cell of reference. Burke & Miller (156) showed that isolated perfused livers from dye-fed rats incorporated 2 to 3 times as much lysine-6-C^{14} into liver and plasma proteins as those from normal rats. Histochemical studies on the loss of RNA and glycogen from the liver during the feeding of 3'-methyl-4-dimethylaminoazobenzene were reported by Griffin and his colleagues (157, 158), and Spain & Griffin (159) studied the levels of biliary pigments. As with other transplanted tumors, a large number of studies have been reported on the enzymatic composition of the hepatoma which has been described and studied by Novikoff (160). This is a highly malignant tumor which has gone through many transplant generations, and it can not be assumed that its properties are necessarily representative of those of the primary liver tumors. However, for some purposes the much greater availability of this tumor as compared to primary neoplasms makes it a useful experimental tool. Because of the limitations of space and our reservations with regard to interpretation of the data in terms of carcinogenesis, the literature on the Novikoff hepatoma has not been included. Much of these data have been considered in the review by Kit & Griffin (161).

Aromatic amines.—Included under this heading are 2-aminofluorene, 4-aminobiphenyl, 4-aminostilbene, 2-naphthylamine, and their derivatives as well as certain metabolites of tryptophan. The knowledge of 2-acetylaminofluorene and related compounds has been comprehensively reviewed by the Weisburgers (162), and the other aromatic amines have been considered by Walpole & Williams (163), Bonser, Clayson, & Jull (164), and Boyland (165).

Considerable attention has been directed to the carcinogenicity of 4-aminobiphenyl and various derivatives [Miller *et al.* (166); Walpole and associates (163, 167); and Morris *et al.* (168)]. Of particular interest are the high activities of 4'-fluoro-4-aminobiphenyl and its N-acetyl derivative for the rat liver and kidney, sites at which the parent compound has little or no activity (163, 166, 167, 168), and the enhancing effect of a 3-methyl or 3-methoxy group on carcinogenic activity (163). The inactivity of 3-hydroxy-4-aminobiphenyl or its N-acetyl derivative when fed to rats (166) is contrary to the suggestion that these compounds are active via *ortho*-hydroxylation *in vivo* (164). Of particular significance are the demonstration that 4-aminobiphenyl is carcinogenic for the bladder of the dog (163, 169) and the strong statistical evidence that it is also carcinogenic for the bladder of man (reviewed in 163, 170). Data have also been presented on the carcinogenicity of certain benzidine (163, 166, 168) and stilbene (163) derivatives for the rat. Bielschowsky (171) showed that, while completely

thyroidectomized rats treated with AF did not develop liver tumors, injection of growth hormone restored the capacity of the liver for tumor development. It is of some interest that a deficiency of growth hormone either as a result of thyroidectomy or hypophysectomy (172) apparently inhibits tumor induction by AF and N,N-diacetyl-AF in the liver but not at all other sites. The observations of Cantarow *et al.* (173) are of interest in this regard. Whereas *in vivo* the livers of rats fed AAF for 90 days incorporated nine times as much uracil-2-C^{14} into RNA as the livers of normal rats, AAF administration to I^{131}-treated rats did not cause a significant increase. However, liver slices from untreated and I^{131}-treated rats fed AAF both incorporated three times as much uracil as slices from normal rats.

The present knowledge of the metabolism of AAF has been reviewed by the Weisburgers (162) who, with Morris, have had a major role in elucidating this problem. These investigators have established that large quantities of the 5- and 7- and smaller amounts of the 1-, 3-, and 8-hydroxy derivatives and conjugated forms thereof are excreted in the urine of the rat (174, 175). 7-Hydroxy-2-acetylaminofluorene is the preponderant metabolite in the guinea pig, which is very resistant to the carcinogenic action of AAF; very little of the other hydroxy derivatives are excreted by this species (176, 177). This difference in metabolism between a susceptible and a resistant species is consistent with the idea that an *o*-hydroxy amine is involved in the carcinogenic action of AAF. Booth & Boyland (178) and Seal & Gutmann (179) showed that 7-hydroxylation of AF and AAF was a TPNH-dependent reaction carried out by rat liver microsomes. In extending these studies Cramer, Miller & Miller (69) observed hydroxylation at each of the 1-, 3-, 5-, and 7-positions when rat liver microsomes were incubated aerobically with TPNH; as with certain aminoazo dye and hydrocarbon-metabolizing systems of the microsomes, the activity of this system was increased severalfold by prior treatment of young rats with MC or BP. The ability of MC to inhibit the carcinogenic action of AAF (121, 122) and to cause increases in the hepatic enzymes which metabolize AAF to *o*-hydroxy amines is difficult to reconcile with the suggestion (176, 177) that the latter derivatives are responsible for the carcinogenicity of AAF. A correspondence between the amounts of 7-hydroxylation and a protein-bound derivative formed on incubation of AAF with rat liver slices was shown by Gutmann *et al.* (180), who suggested that it is an oxidized derivative (such as a quinone imine) which combines with the protein. More recently Nagasawa *et al.* (181) have suggested that 1-hydroxy-2-aminofluorene may be similarly involved. However, the differences between the protein-bound aminoazo dyes formed *in vivo* and *in vitro* (136) emphasize the difficulties that may be encountered in relating the *in vitro* binding of AAF to the combination of AAF or a metabolite with rat liver protein *in vivo*. Gutmann & Peters (56) observed that C^{14}-labeled AAF and AF applied to the skin of rats were absorbed rapidly, but the carcinogens were apparently not metabolized by

the skin. The deacylation of AAF has been studied by Nagasawa & Gutmann (182).

Kielley (183, 184, 185) and Emmelot & Bos (186, 187) have studied the inhibition by AAF, N,N-diacetyl-2-aminofluorene, certain aminoazo dyes (e.g., DAB, 3'-methyl-4-dimethylaminoazobenzene), and carbon tetrachloride of the DPN-linked oxidation of glutamate by rat and mouse liver mitochondria. The fluorene carcinogens are strong competitors of DPN for the glutamic dehydrogenase of mitochondrial extracts (184, 186), and Emmelot & Bos (187) postulated that the sequence of events involved a swelling of the mitochondria under the influence of the carcinogens with a dislocation of the DPN and an activation of latent ATPase. The possible importance of the impairment of glutamate oxidation in carcinogenesis has been discussed by Kielley (185). Roth (188) observed a depression of the level of alkaline RNAase of liver mitochondria and blood serum of rats fed AAF.

Bonser and her associates (164) and Boyland (165) have recently reviewed their work on the carcinogenicity of various compounds for the urinary bladder of the mouse by means of the bladder implantation procedure. This ingenious and useful method has two deficiencies: (a) appreciable incidences of bladder tumors develop in mice receiving implants only of the vehicle (especially cholesterol), and (b) even the most active compounds yield tumor incidences of less than 50 per cent. Since aromatic amines are generally inactive or have only low activity under these conditions while related o-hydroxy derivatives in some instances are considerably more active, this work suggests that aromatic amines are carcinogenic by virtue of their *ortho*-hydroxylation *in vivo*. Bonser and her associates have been especially interested in derivatives of 2-naphthylamine and 4-aminobiphenyl, both of which induce bladder tumors in dogs and man, while Boyland's group has also considered the possible role of the o-hydroxyamines formed from trytophan. Two metabolites, 3-hydroxykynurenine and 3-hydroxyanthranilic acid, and 2-amino-3-hydroxyacetophenone, which has been implicated as a metabolite, are active when implanted in the mouse bladder and have been suggested as etiological factors in human bladder cancer. Both Boyland & Williams (189) and Brown, Price & Wear (190) found that many bladder-cancer patients excrete abnormally high amounts of several metabolites of tryptophan in the urine. Boyland et al. (191) have also considered the effect of alterations in the level of urinary β-glucuronidase on the levels of free o-hydroxy amines in the bladder, and Elson et al. (192) suggested that carcinogenic amines may be excreted chiefly as glucuronides and noncarcinogenic amines primarily as sulfates of phenolic metabolites. However, the observation that certain o-methoxyamines are as active as the corresponding hydroxyamines raises the question of the role of the hydroxy group (163, 193). While it is possible that the methoxy group is cleaved *in vivo* to yield the hydroxyamine (194, p. 430), it may be

that the hydroxy group is but one of several substituents which can activate some other essential site on the molecule [Clayson, Jull & Bonser (193)]. In the latter case *ortho*-hydroxylation would not be a unique requirement for carcinogenic activity with this class of amines. The association of appreciable radioactivity with the urinary bladder, even after extensive washing, of several species of animals given 2-naphthylamine-5,8-C^{14} is of some interest (195, 196) and may indicate a protein-bound derivative. Booth & Boyland (178) have studied the TPNH-dependent hydroxylation of several aromatic amines by rat liver microsomes, and Boyland & Manson (197) extended their studies on the urinary metabolites of 2-naphthylamine.

Hormones.—The roles in carcinogenesis of exogenous hormones and of hormonal imbalances have been the subjects of a number of reviews. Thus, various aspects of the over-all problem have been considered by Shimkin (13), Gardner (198), Bielschowsky & Horning (199), and Lacassagne (200). Mühlbock (201) examined the literature on the role of hormones in mammary carcinogenesis, while Cantarow (202) discussed endocrinological aspects of liver disease and experimental liver tumors. The hormonal factors involved in the induction of thyroid tumors by low-iodine diets were considered by Leblond, Isler & Axelrad (203); excessive thyrotrophic hormone output appears to be indispensable for the formation of the type-β nodules as well as for their maintenance. Prolonged administration of 5-iodo-2-thiouracil caused thyroid tumors in rats, but was less active than thiouracil [Money *et al.* (204)]. Furth & Clifton (205) have studied the development of pituitary tumors with specific hormonal characteristics. Thus, thyrotrophic tumors arise as a result of a sustained lack of thyroid hormones, adrenotrophic tumors arise after x-radiation of the pituitary gland region, and mammotrophic tumors arise after x-radiation or estrogenic treatment. The secretion of hormones by these tumors, their dependent status in early transplant generations, and their transition to autonomous tumors are of considerable interest. The tumorigenic responses of the hamster to estrogen or combined estrogen-androgen treatment have been summarized by Kirkman (206). In addition to the well-known renal tumors, which have also been studied by others [e.g., Horning (207)], tumors arise from the uterine endometrium, from the muscularis of the vas-deferens-epididymal tail, and from the flank organ. Endometrial hyperplasia, polyps, and a few carcinomas were induced in rabbits by strong estrogenic stimulation; the similarity of these lesions to those developing spontaneously in women was stressed [Meissner, Sommers & Sherman (208); Sommers & Meissner (209)]. BALB/c male mice implanted with stilbestrol-cholesterol pellets developed a high incidence of interstitial cell tumors of the testis; the hormonal dependence of these tumors was studied at various stages of development [Andervont, Shimkin & Canter (210)]. On the basis of the known relationships between certain hormonal imbalances and the development of endocrine tumors, Crile (211) developed a speculative theory of the genesis of tumors at all sites.

Ethionine.—The carcinogenicity of ethionine in the diet for rat liver has been definitely established by Farber (212, 213) and confirmed by Gelboin, Miller & Miller (131). Farber (212) has shown the similarities in the sequence of histological changes in the liver, whether induced by ethionine, AAF, or 3'-methyl-4-dimethylaminoazobenzene. Dietary methionine and, to a lesser extent, choline and betaine inhibited the action of ethionine, while homocystine in the diet greatly enhanced the formation of hyperplastic nodules in the liver by ethionine [Farber & Ichinose (214, 215)]. Many of the tumors appeared to arise in these nodules. The interrelationships between choline deficiency, ethionine, and methionine in liver tumor induction have been investigated by Salmon & Hare (216). In view of the incorporation of ethionine into liver protein (217) and recent studies showing the incorporation of other amino acid analogues into the proteins of various biological systems, it seems likely that some of these analogues would prove to be carcinogenic if administered in high amounts for a sufficiently long time.

Urethan.—Although urethan had been considered for many years to be exclusively a lung carcinogen, it is now known to have a much wider action. Administered orally, parenterally, or cutaneously, urethan initiates skin carcinogenesis in mice; subsequent application of a promoting agent is essential for the gross manifestation of tumors (16, 218 to 221). Multiple papillomas of the forestomach were found by Berenblum & Haran-Ghera (222) in mice given large oral doses of urethan, while Tannenbaum & Silverstone (223) observed that five separate lesions—mammary carcinomas, malignant mesenchymal tumors, lung adenomas, cystadenomas of the lacrimal gland, and blood cysts of the liver—were either evoked or potentiated by long-term application of large amounts of urethan cutaneously. Kawamoto, Ida, Kirschbaum & Taylor (224) showed that, while not leukemogenic for low-leukemia strains of mice when administered alone, urethan markedly augmented the leukemogenic action of x-rays, estrogenic hormone, or MC.

From studies on the inhibition of lung tumor induction in mice by the administration of various components or precursors of nucleic acids just prior to dosage with urethan, Rogers (225) suggested that the locus of action of urethan is in the synthesis of nucleic acids and is initiated at or about the level of ureidosuccinic acid. This locus of action was deduced from the relative activities of various nucleic acid derivatives, with orotic acid being the most active inhibitor *in vivo*, and it was supported by the structural similarity of urethan to ureidosuccinic acid. The latter argument may not apply since Rogers (226) later showed that the leukemogenic action of MC is also inhibited by orotic acid. Studies on the distribution of radioactivity from carbonyl- or ethyl-labeled urethan have been reported by Berenblum *et al.* (227).

Carbon tetrachloride.—The hepatocarcinogenicity of carbon tetrachloride for mouse liver has been repeatedly demonstrated, and Andervont (228)

has recently studied the susceptibility of C3H mice to this agent. Recknagel and his associates (229, 230), Calvert & Brody (231), and Frunder *et al.* (232) suggested that the primary biochemical lesion is an impairment of oxidative phosphorylation and a loss of pyridine nucleotide oxidations because of a loss of mitochondrial integrity, while Decsi *et al.* (233) reported a decreased capacity for the transfer of phosphate from ATP to coenzyme A. Thiers & Reynolds (234) found a striking increase in the calcium content of carbon tetrachloride-damaged mitochondria, as well as alterations in the content of other metals. Disruption of mitochondrial function may be an important biochemical link relating the actions of AAF (184 to 187), aminoazo dyes, carbon tetrachloride, and thioacetamide (235) in the liver.

Alkaloids.—In continuing their investigations on the Senecio alkaloids, Schoental and her associates (236, 237) demonstrated that the liver lesions (including tumors) produced by the repeated administration of riddelline, retrorsine, and isatidine persisted in the absence of the alkaloids and that even a single oral dose of lasiocarpine caused persistent liver damage.

Thioacetamide and thiourea.—Gupta (238) extended the earlier observations on the hepatotoxic action of thioacetamide; metastatic liver tumors were found in four of five rats fed the compound more than 47 weeks. In acute intoxication by thioacetamide the significant biochemical lesion appeared to be an altered permeability of the cell wall with a resultant accumulation of calcium and an inhibition of certain mitochondrial enzymes [Gallagher *et al.* (235)] Grant & Rees (152) showed that various oxidative systems of liver mitochondria from rats fed thioacetamide for several weeks were much more adversely affected by "aging" than those of normal rats; the activities were restored by fortification with DPN, versene, and coenzyme A. A high percentage of rats treated with thiourea for one to two years developed carcinomas in the region of the external auditory duct and in the glands of the eyelids [Rosin & Ungar (239)].

Quinoline derivatives.—The carcinogenicity of compounds related to 4-nitroquinoline-N-oxide have been studied by Nakahara, Fukuoka, and their associates (240, 241); when applied cutaneously to mice 4-nitroquinoline-N-oxide has an activity similar to that of 1,2,5,6-dibenzathracene. The nitro and N-oxide groups are both necessary for activity. Since the nitro group is easily replaced by —SR of RSH compounds, it seems likely that protein-binding of this carcinogen takes place. The carcinogenicity of 8-hydroxyquinoline for the urinary bladder of mice was demonstrated by Allen *et al.* (242); it did not cause tumors on repeated intratesticular injection into hamsters [Umedo (243)].

Cholesterol.—Kennaway (244) has recently reviewed the literature on the carcinogenicity of cholesterol. Highly purified preparations of this sterol exhibit weak sarcomagenic activities. Hieger (245) has assembled his data on this compound; of 1434 mice which received subcutaneous injections of cholesterol dissolved in olive oil or lard, 70 developed sarcomas at the

site of injection. On the other hand, Bischoff (246) obtained no sarcomas by the injection of sesame oil solutions of cholesterol in Marsh Buffalo mice. However, certain derivatives—4-cholestene-3,6-dione, 5α, 6α-epoxy-3β-cholestanol, and 6β-hydroperoxy-4-cholestene-3-one—induced sarcomas when injected in sesame oil solution but not when administered as aqueous colloids; a number of other derivatives were inactive. Cholesterol pellets have weak carcinogenic activity when implanted in the urinary bladders of mice (193).

Plastics.—Further histological and chemical studies on the sarcomagenic action of plastics in rats have been reported by Oppenheimer *et al.* (247) and Danishefsky *et al.* (248); Bering & Handler (249) found sarcomas in two of 50 hamsters implanted with polyethylene. Two epoxy resins were observed to induce low incidences of skin carcinomas or sarcomas in mice [Hine *et al.* (250)].

Other organic compounds and complex mixtures.—Bekemeier, Hannig & Pfennigsdorf (251) were unable to confirm earlier reports of the carcinogenic activity of dulcin (*p*-ethoxyphenylurea) in rats fed high levels for up to 660 days. Allylmethanesulfonate, 1,4-dimethane-sulfonoxy-2-butyne, and the *cis* and *trans* isomers of 1,4-dimethanesulfonoxy-2-butene, when administered with croton oil, induced papillomas in the skin of mice [Roe (252)]. Dimethylnitrosoamine was shown by Magee & Barnes (253) to induce severe liver damage and hepatic tumors in rats, but not in rabbits, fed 50 p.p.m. for 26 to 40 weeks. The compound is rapidly metabolized by rabbits, rats, and mice [Magee (254)].

Four reports have appeared of the carcinogenicity of complex mixtures. Szepsenwol (255) found malignant tumors in 12 of 16 mice fed for nearly two years on a laboratory chow diet supplemented with hard-boiled eggs; two of 16 control mice developed tumors. Blomqvist (256) observed malignant abdominal sarcomas in 19 of 30 rats injected twice weekly over a 13-month period with dried regenerating liver from partially hepatectomized rats. Fortner (257) found malignant tumors of the intestinal tract in four hamsters which received repeated injections of bile from human patients with cancer of the extrahepatic biliary tract. Rats which were fed a low-protein diet and were treated orally or intravaginally with certain spermicidal contraceptives developed a high incidence of tumors at various sites, especially the mammary gland and the liver [Hoch-Ligeti (258)]. Apparently both the diet and the contraceptives were contributory factors since rats fed the low-protein diet alone had appreciable incidences of mammary tumors as well as liver damage and since rats fed the contraceptives in a more adequate diet did not develop tumors. The possible importance of the 8-hydroxyquinoline content of the contraceptives was noted by the author; this compound has induced tumors in the urinary bladders of mice (242). The significance of this group of experiments is difficult to judge; confirmation and further data are needed.

Inorganic materials.—The present knowledge of the roles of asbestos,

hematite, and chromium and nickel compounds as carcinogenic agents for man has been reviewed by Goldblatt (259). Hueper (260) has studied the pulmonary lesions (including tumors) inducted in guinea pigs and rats by the long-continued inhalation of finely powdered metallic nickel, and Grogan (261) examined the fate of fine chromium particles introduced directly into the lungs. Like films of various polymers (247), films of certain metals also induce low incidences of sarcomas after long latent periods when implanted subcutaneously in rats (262, 263).

<div align="center">CARCINOGENIC VIRUSES AND SUBCELLULAR PARTICLES</div>

The roles, known and projected, of viruses in the etiology of tumors and the properties of the known tumor viruses have received much attention in the past few years. A meeting of the New York Academy of Sciences considered "Subcellular Particles in the Neoplastic Process" (264) while "Viruses and Tumor Growth" was the topic for a symposium at the University of Texas (265). Critical reviews on the known tumor viruses and the implications of these findings with regard to other tumors have been provided by Harris (9), Furth & Metcalf (8), and Dmochowski (266). Electron microscopy of tumor virus preparations and of tumor cells has been reviewed by Bernhard (267) and by Dmochowski & Grey (268). The reader is referred to these sources for comprehensive consideration of the subjects.

Rous sarcoma virus.—The problems involved in the purification of the Rous sarcoma virus were discussed by Bryan & Moloney (269), whose best preparations were about 1 per cent pure. Recently Epstein (270, 271) has reported that preparations of the Rous virus grown on the chorioallantoic membrane and isolated at a fluorocarbon-water interface are quite uniform as studied by electron microscopy and appear to be relatively free of host contaminants; this material was infective. RNAase treatment altered the structure of the particles. Digestion of extracts of Rous sarcoma tissue with RNAase or DNAase had no effect on their infectivity [Bielka & Graffi (272)]. The infectivity of Rous sarcoma extracts was strongly inhibited by low concentrations of cetyltrimethylammonium bromide; this effect was largely overcome by the addition of crude RNA preparations [Guerritore (273, 274)]. Moloney (275) showed that the inhibition of the infectivity of the Rous virus by oxidation products of various lipides was minimized by the use of citrate rather than phosphate buffers. Other data on the Rous virus have been reviewed by Rubin (276) and in the more inclusive reviews listed earlier.

Avian leukosis viruses.—The etiology of the avian leukoses and the isolation and properties of the myeloblastosis and erythroblastosis viruses from chicken plasma have been reviewed by Beard (277, 278), while the modes of transmission of the leukoses under natural conditions have been considered by Burmester (279, 280). While the viruses of visceral lymphomatosis, myeloblastosis, and erythroblastosis are distinct, they constitute members of a family of viruses which parasitize the various stem cells of the hem-

atopoietic system (277, 278). From immunological studies these viruses appear to be part of a larger family of related viruses which includes those responsible for the transmissible avian sarcomas. Beard and his associates earlier showed that ATPase is an integral part of the virus of avian myeloblastosis. On the other hand, recent work from the same laboratory has demonstrated that the virus of erythroblastosis contains insignificant amounts of this enzyme [Bonar *et al.* (281)]. The erythroblastosis virus also differs from the myeloblastosis virus in that it does not respond to Forssman antibody; both viruses contain components antigenically similar to chicken protein intrinsically incorporated in the infective particles [Beard *et al.* (282)]. Both viruses are most stable near pH 7, but the erythroblastosis virus is significantly more stable at low temperatures ($-78°$) (283). Electron microscopy of spleens from chickens with erythroblastosis revealed characteristic particles not found in normal tissues [Dmochowski *et al.* (284)].

Mouse mammary tumor agent.—The recent advances in the knowledge of the mouse mammary tumor agent have been reviewed by Bittner (285, 286). Pitelka *et al.* (287) observed viruslike particles similar to those previously demonstrated in some mouse mammary tumors [see reviews by Dmochowski & Grey (268) and Bernhard (267)] in the hyperplastic alveolar nodules of the mammary glands of C3H/He mice; the positive identification of these particles as the mammary tumor agent is still not possible. A variant which causes mammary tumors to develop approximately two months earlier than with the parent strain has been studied (288).

Rabbit fibroma and myxoma viruses.—Kilham (289, 290) demonstrated the transformation of rabbit fibroma virus into myxoma virus by inoculation of tissue cultures simultaneously with heat-inactivated myxoma and live fibroma viruses. This transformation, which has previously been observed *in vivo*, was dependent on an active proliferation of the tissue cultures. A lesser species specificity of these viruses for growth in tissue culture than *in vivo* was observed (291). Treatment of domestic rabbits with x-radiation or certain hydrocarbons prior to the administration of fibroma virus altered tumor development so that, as with the fibromas of wild rabbits, they could be passed to other rabbits through mosquitoes [Dalmat (292)].

Rabbit papilloma virus.—Noyes & Mellors (293, 294), who used fluorescent antibody techniques, demonstrated papilloma virus antigens in the nuclei of differentiating cells of the keratohyaline layers and in the keratinized layers in the papillomas of wild rabbits, but not in the deeper proliferating epithelial cells. These antigens were present in very minute amounts in the papillomas of domestic rabbits. The authors postulated that in the papillomas of domestic rabbits the virus consisted mainly of nucleic acid and lacked the protein which confers antigenicity and helps preserve its infectivity in the virus from wild rabbits. Direct evidence for such a "protein-deficient" virus is lacking, however.

Mouse leukemia and related agents.—Leukemia occurs as a spontaneous

disease in mice of various strains, and Gross was the first to show that in some cases these primary leukemias, or transplants of them, can be transferred to newborn mice of the same or different strains with cell-free preparations. In some mice parotid gland tumors or sarcomas were induced. The incidences of both leukemia and the solid tumors may be quite low, and the latent periods are long. The work of Gross, its confirmation and extension by other investigators, and other aspects of the problem have been adequately reviewed by Gross (7, 295, 296). In recent work by continued subculturing and selection, Gross (297) has obtained much more virulent strains which induce high incidences of leukemia in three months when injected into young or adult mice; parotid gland tumors were found only in mice injected when very young. Active cell-free preparations from x-ray-induced leukemias have yielded low incidences of leukemia (298). Stewart, Eddy, and their associates (10, 11, 299) have propagated in tissue cultures a mouse leukemia-derived parotid tumor agent which has greater virulence, induces tumors in mice not only of the parotid gland but also at a number of other sites, causes similar tumors to arise in hamsters, and has various characteristics indicating its viral character. Furth *et al.* (300) showed that of the leukemias induced by cell-free filtrates some had the genetic characteristics of the strain used as the source of inoculum, some had the genetic characteristics of the recipient mouse, and some had genetic components from both sources. For this reason they suggested the possible roles of transduction or transformation processes in leukemogenesis by these cell-free preparations. Hays, Simmons & Beck (301) and Latarjet *et al.* (302) have reported preliminary data on the induction of mouse leukemia with nucleic acid preparations from lymphoid tissue of leukemic or nonleukemic AKR (high leukemia strain) mice.

These studies stimulated others to search for cell-free agents. Graffi and his associates (303, 304) showed that the injection of cell-free preparations of certain transplantable mouse sarcomas and carcinomas into young or adult mice precipitated the development of myeloid leukemia in one-half or more of the mice after a latent period of several months; these results were confirmed by others (305, 306). These tumors could be further transplanted by cell-free preparations, but not by cells. The activity of the agent was unaffected by glycerination (307) and was stable in crude preparations to RNAase, DNAase, and trypsin (308). In preliminary fractionations ATPase activity appeared to be associated with the leukemia-inducing activity, but the preparations were too crude for conclusive results (309). As with the Gross leukemias, electron microscopy revealed the presence of particles which may be the virus (310). The agent appears to be active on injection into newborn rats (311). Fey (312) reported changes in the blood of treated mice within a few days after inoculation which progressed until leukemia developed several months later. Somewhat puzzling are the results of Graffi (304) Schmidt & Lohmann (306) and Gross (313) on increased incidences of leukemia in mice treated with various nontumor preparations as compared

to untreated controls; the percentage of leukemic mice was, however, considerably lower than for mice treated with extracts of leukemic tissues. These results raise the question of whether there is a rather widespread occurrence of leukemic agents or whether latent agents in some mice are easily activated.

Friend (314, 315) obtained leukemia by injection of a cell-free extract from the spleen of a mouse treated in infancy with a similar extract of an Ehrlich ascites tumor. The disease was readily passed to adult mice by cell-free preparations, but not by cellular transplants; the average survival time of the mice was two months. The agent retained its infectivity after massive doses of x-radiation and after storage at low temperatures. Electron microscopy revealed intracellular particles in about one-fourth of the sections (316). Virus production in tissue culture was low (317). The pathology of this leukemia has been described by Metcalf, Furth & Buffett (318).

Schwartz, Schoolman, and their associates (12, 319, 320, 321) have studied the development of leukemia in adult AKR mice injected with cell-free preparations of brain from human patients or mice with leukemia. Bergol'ts (322) has similarly stimulated the development of leukemia in mice by injection of cell-free extracts of human leukemic tissue.

COCARCINOGENESIS

Cocarcinogenesis, particularly with regard to the induction of skin tumors, has been considered in an excellent review by Salaman (16). While the concept that tumors may be "initiated" by one agent and "promoted" by another has received rather general support, the biochemical alterations involved at each stage have received little study. One point of interest is the action of urethan, which apparently initiates the neoplastic transformation of mouse skin so subtly that no histological changes are discernible (323). Croton oil, widely used as a promoting agent and in earlier work considered to be inactive as an initiator, has induced some papillomas without prior application of a hydrocarbon (324). Sicé (325) has fractionated croton oil into at least two different tumor-promoting principles and has studied their chemical properties. Boutwell & Bosch (326) confirmed the observations of Setälä, Holsti & Lundbom (327) that various nonionic surface-active agents will cause the development of papillomas initiated by hydrocarbons and also obtained some papillomas in mice treated only with these agents. The histological changes in skin treated with the surface-active agents were studied by Dammert (328) and by Setälä & Stzernvall (329); the latter investigators suggested that these agents act through alterations in the permeability of the cell and nuclear membranes. The induction of "tumors" in the slime mold physarum by a combination of methylcholanthrene and Tween 80 has been reported [Setälä et al. (330)], but the relationship of such tumors to those of animals is unknown. Phenol (331, 332) and certain solvents with long alkyl chains (e.g., n-dodecane and dodecylbenzene) [Horton et al. (333)] also promote the development of tumors initiated by polycyclic hydrocarbons. The

idea that not only certain chemicals, but also viruses, can act as cocarcino-
gens have been emphasized by Furth & Metcalf (8). The studies of Duran-
Reynals (334) on the development of skin tumors in mice treated with
methylcholanthrene, vaccinia virus, and cortisone furnish one possible
example.

THEORIES OF CARCINOGENESIS

As in preceding years, a number of investigators have attempted to
synthesize the data on carcinogenesis and the properties of normal and ma-
lignant cells into theories concerning the nature of the malignant trans-
formation. An over-all assessment of this subject is not possible here, but a
number of recent papers should be mentioned. The over-all problem of
carcinogenesis has been discussed in clear and thoughtful papers by Burnet
(3) and by Shimkin (73), and some aspects of this general problem have
also been considered by Griffin (335). The various mechanisms which have
been suggested for the action of chemical carcinogens have been critically
reviewed by Haddow (336), while Furth & Metcalf (8) and Upton (37)
have similarly considered possible means by which viruses and radiations,
respectively, may induce tumors. The deletion hypothesis of carcinogenesis
was restated by Potter (337) in current biochemical terms; Osgood (338)
presented a somewhat different theory which, however, also assumes a loss
of enzymatic functions controlling growth. Rose (339), on the other hand,
has suggested that cancer may involve a gain in self-perpetuating abnormali-
ties in differentiation. An immunological basis for the origin of cancer has
been the subject of several articles by Green (340, 341, 342), and Crile
(211) has based a theory on endocrinological imbalances. Orr (343) has
reviewed data suggesting that chemical carcinogenesis in skin may be an
indirect or "field" effect. Mutations as a basic cause of cancer have received
considerable attention, particularly through a recent symposium on this
subject (344) and articles by Fisher (345) and Armitage & Doll (346).

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CLINICAL BIOCHEMISTRY[1,2]

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Since it is possible to cover only a small part of current work on the biochemistry of disease in a review of 20 pages, the authors have selected a few subjects which may be of special interest. They are well aware that their treatment of even these subjects is far from complete.

Diabetes and Insulin

For several years rat diaphragm has been much used as an indicator of insulin activity. The work of Shaw & Stadie (1), who have shown the co-existence of an insulin-responsive and an insulin-nonresponsive glycolytic system in rat diaphragm, is therefore of great theoretical and practical interest. The metabolism *in vitro* of rat diaphragms in a phosphate-saline medium with added glucose-U-C^{14} was reported. Total lactic acid formation from glucose was unaffected by addition of insulin to the medium; in contrast, glycogen synthesis from glucose in the medium was always increased. It was also shown that insulin increased the turnover rate of glucose-6- and glucose-1-phosphates, but, surprisingly, C^{14} was never found in the fructose-1,6-diphosphate. It was also shown that there was a free exchange between the esters formed in the insulin-nonresponsive system and esters in the medium, whereas the esters formed in the insulin-responsive system never left the cells. Shaw & Stadie conclude that the insulin-nonresponsive system, by which glucose is broken down to lactic acid, probably is situated on the cell surface, whereas the other system is located in the interior of the cell. This system builds up glycogen from added glucose. According to Shaw & Stadie, the probable function of insulin has nothing to do with hexokinase, but insulin in some way facilitates the penetration of glucose into the cell and so makes it possible for the glucose molecules to reach the enzymes of the glycogen-forming system.

The insulin antagonists of human serum have been further studied. Berson & Yalow (2) have analyzed the sera of nine insulin-treated subjects for insulin-binding antibody. The sera were fractionated with cold ethanol, according to the Cohn procedure, and the fractions were tested for the presence of insulin-binding antibody by means of I^{131}-labelled crystalline beef insulin and paper strip electrophoresis. In all cases the antibody was present

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[1] The survey of the literature pertaining to this review was completed in November 1958.

[2] The following abbreviations are used: DOPA for dihydroxyphenylalanine; DPN for diphosphopyridine nucleotide; TPN for triphosphopyridine nucleotide (oxidized form), TPNH (reduced form); UDP for uridine diphosphate; UTP for uridine triphosphate.

in fraction I + III (α- and β-globulins). The antibody-insulin complex migrated in the inter-gamma-beta region. Berson & Yalow (3) have studied also the kinetics of the reaction between insulin and insulin-binding antibody, using crystalline beef insulin-I^{131} as a tracer. Insulin and antibody reacted to form a nonprecipitating complex which was in reversible equilibrium with the uncomplexed species. Steady-state studies indicated that insulin was univalent in the reaction but that two distinct orders of antibody binding sites were demonstrable in most antisera. The demonstration of cross reactions between beef and pork insulin suggested that the two orders of antibody binding sites observed may have been caused by different affinities of each of the species-specific antibodies for beef insulin. The maximal insulin-binding capacity rarely exceeded 10 units per liter in sera from nonresistant subjects but ranged from 80 to 400 units per liter in sera from four subjects with insulin resistance. Finally, Yalow & Berson (4) have shown that the apparent inhibition of liver insulinase activity by serum containing insulin-binding antibody simply results from the complexing of insulin by the antibody. Field & Stetten, together with Tietze (5), have continued their studies of the insulin antagonist in the serum of patients in diabetic acidosis. In starch block electrophoresis it migrated with the α_1-globulin fraction. The antagonist did not exhibit any glucagon-like activity. It was capable of inhibiting human insulin as well as beef-pork insulin in the diaphragm system. The mechanism of action of this antagonist is not clear.

Lipide metabolism and ketosis in diabetes.—Brady, Mamoon & Stadtman (6) and Langdon (7) have published results of experiments on pigeon and rat livers which seem to indicate that the synthesis of fatty acids occurs only in the soluble cytoplasm, while the oxidation seems to take place in the mitochondria. Both Brady *et al.* and Langdon have found evidence that TPNH is necessary for this synthesis, and Langdon has concluded from his experiments that impairment of lipogenesis, which characteristically accompanies diabetes mellitus, may be attributable in part to a decreased availability of TPNH in the extramitochondrial portion of the liver cell. Siperstein & Fagan (8, 9) have confirmed the findings of Brady *et al.* and Langdon and have proceeded still further. They studied the synthesis of cholesterol and fatty acids in cell-free homogenates of normal and alloxandiabetic rat livers. When glucose was added to the medium, there was a pronounced stimulation both in the synthesis of fatty acids and in the synthesis of cholesterol from acetate upon the addition of TPN. The addition of DPN, on the contrary, had only a small effect. Now, DPN is necessary for the metabolism of glucose via the Embden-Meyerhof pathway, while TPN stimulates the hexosemonophosphate shunt. When both coenzymes were added, there was a further stimulation in the synthesis of fatty acids, but the synthesis of cholesterol was depressed in relation to that seen when the shunt alone was stimulated. Isocitrate together with TPN had the same effect as glucose and TPN. As it is well known that isocitrate enters a metabolic pathway which leads to the reduction of TPN, it seems as if the available amount of TPNH should be the limiting factor both in

the synthesis of fatty acids and in the synthesis of cholesterol. Siperstein & Fagan also think that the same mechanism, i.e., lack of reduced TPN, might explain the accumulation of ketone bodies, β-hydroxybutyric and acetoacetic acids; the CoA derivatives of these two acids are among the fatty acid precursors which precede the step in fatty acid synthesis which, according to the work of Langdon (7) requires TPNH as a coenzyme. Lynen *et al.* (171) have also made a very interesting contribution to the explanation of ketogenesis.

Blood sugar and renal clearance of phosphate.—Huffman *et al.* (10) report that the blood glucose level exerts a powerful influence on the renal clearance of phosphate. The action is more pronounced in hypo- than in hyperglucemia.

CONGENITAL GALACTOSEMIA

The brilliant work of Kalckar and his team in 1956, which proved that congenital galactosemia is caused by the lack of a single enzyme, galactose-1-phosphate transferase, has led to a better understanding of the metabolism of galactose in man and of the mechanism of congenital galactosemia.

Since then there has been increased interest in this inborn error of metabolism. Kalckar's team has published a new series of papers (11 to 16), but the work which may prove to be of the greatest clinical interest comes from one of the group, Isselbacher (17, 18).

Isselbacher studied galactose metabolism in an adult patient with galactosemia and could show conclusively that, in spite of a lack of the specific enzyme galactose-1-phosphate transferase, this patient could convert galactose to glucose. When looking for an explanation for this puzzling fact, Isselbacher found in the nuclei of mammalian liver cells a new enzyme which he calls UDP-galactose pyrophosphorylase. This enzyme catalyzes the reaction:

$$\text{Gal-1-P} + \text{UTP} \xrightleftharpoons[\text{UDPGal} + \text{PP}]{\text{UDPGal pyrophosphorylase}} \tag{1}$$
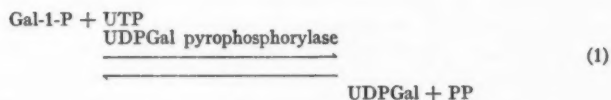
The UDP-galactose formed via this pathway is then converted to UDP-glucose and in turn to glucose-1-phosphate by means of the reactions previously described. The interesting thing now is that the liver of new-born animals—and the same seems to be true in experiments performed on human livers—is poor in both galactose-1-phosphate transferase and in UDP-galactose pyrophosphorylase. In the adult animal, however, UDP-galactose pyrophosphorylase increases considerably, and this may be the reason patients with congenital galactosemia develop their most pronounced symptoms in infancy. Subsequent increase in activity of the UDP-galactose pyrophosphorylase may explain the improved galactose metabolism which occurs in these patients, despite the continued lack of galactose-1-phosphate transferase. These findings stress the importance of adequate treatment of

all children with congenital galactosemia, as it seems possible that their galactose metabolism, when they have grown up, might be adequate for the normal needs of the organism.

DISEASES INVOLVING THE METABOLISM OF AMINO ACIDS

Plasma amino acid concentrations.—By paper chromatography Mc-Menamy *et al.* (19) determined concentrations of 18 individual amino acids, unbound, in plasma samples from 15 normal subjects. With the exception of tryptophan and glutamic acid, agreement with other assays was obtained. At physiological pH tryptophan is bound to a nondialyzable plasma substance, probably a protein. Recovery studies of amino acids added to plasma also showed that none were bound except tryptophan. Plasma levels of amino acids of patients in good nutritional status dropped an average of 34 per cent immediately after surgery. In similar studies, Salisbury *et al.* (20) measured free plasma amino acids with microbiological methods on a pooled plasma sample of nine fasting young men and on individual plasma samples of six patients with severe uremia. In five of six uremic plasma specimens, the content of free arginine, glycine, and proline was definitely elevated.

The amino acids in blood plasma and urine during pregnancy (third and fourth months) were studied by Christensen *et al.* (21) with ion exchange resin chromatography. The concentration of plasma amino acids was generally found to be lower than in the nonpregnant state. It is likely that the lowered concentration of the amino acids in plasma results in part from the excretion of larger amounts of amino acids by the kidney.

In liver disease, individual amino acids were studied by Iber *et al.* (22). The plasma amino acids rose with increasing severity of the liver disease but were not always elevated in hepatic coma. Methionine and tyrosine were disproportionally elevated, and this was the most common abnormality in the individual amino acids found in all of the four patients with liver coma. Disturbances in amino acid metabolism have also been shown, by analysis of free amino acids in plasma, to occur in leukemic individuals (23). With improved and simpler methods, analysis of blood plasma for amino acids promises to become an important field for the clinical chemist.

Aminoaciduria.—An increasing number of diseases are now known to be associated with changes in the amino acid composition of urine. These changes may be sufficiently gross to be readily detectable on ordinary two-way paper chromatograms. Thus Allan *et al.* (24, 25) have found a large excretion of a single unidentified amino acid or peptide in a certain form of mental deficiency in children. The metabolite in question has since been identified as argininosuccinic acid (26). DeVries *et al.* (27) have found an excessive excretion of glycine in four members of a family and in three members with nephrolithiasis. The glycinuria was caused by a renal mechanism, and was not associated with defective reabsorption of other amino acids or of phosphate or glucose. A kidney stone obtained from one of these patients was composed mainly of calcium oxalate and contained a

small amount of glycine present in nonprotein, nonpeptide form. Doolan *et al.* (28) have made simultaneous measurements of the renal clearances of inulin and lysine in cystinuria. A very small amount of lysine is reabsorbed from the glomerular filtrate, and the renal tubules are unable to reabsorb any additional amount; on loading with lysine, their clearance value approaches that of inulin. Therapeutic efforts directed at the specific tubular defect appear so far to hold little promise. However, the decrease in cystine excretion which can be accomplished by dietary restrictions should not be neglected (29). The aminoaciduria in Cooley's sickle cell anemia has been studied by Choremis *et al.* (30). Cusworth (31) has isolated phosphoethanolamine in pure form from the urine of an adult case of hypophosphatasia.

Diseases Involving the Metabolism of Plasma Proteins

Albumin.—Nennstiel & Becht (32) have reported a hereditary anomaly in which two different fractions of albumin determined by paper electrophoresis are present in blood plasma. The mechanism which causes hypoalbuminemia in patients with ulcerative colitis and regional enteritis is discussed by Steinfeld *et al.* (33). Although in these conditions there might be a leakage of all serum proteins, mainly albumin escapes into the gut.

α-Globulins.—The physiological implications of the thyroxine-binding α-globulin of human serum have been studied by Robbins and co-workers (34, 35, 36). Studies on haptoglobulin, the hemoglobin-binding serum protein which migrates with the α_2-fraction, are reported by Nyman (37) in pernicious anemia, and by Laurell & Nyman (38) in hemoglobinemia.

"U" protein.—Paper electrophoresis of serum from children with liver and kidney disease has led to recognition of a protein which migrates between the α_2- and β-globulins (39). This protein has been observed by other workers, but, because of confusion in nomenclature, the new designation "U" protein, has been proposed by Stern *et al.*

γ-Globulins.—Martin *et al.* (40) have studied the metabolism of injected pooled human γ-globulin into four hypogammaglobulinemia patients. The injected γ-globulin is distributed in an apparent fluid volume of approximately 12 per cent of body weight, and in each patient individual antibodies derived from the pool are degraded at different rates. The mean half lives of individual antibodies in the group varied from 21.7 to 44.9 days, while that of pooled γ-globulin was 32.6 days. Martin *et al.* (41) have also studied the antibody-protein synthesis by lymph nodes after subcutaneous homotransplantation to a hypogammaglobulinemic adult.

Wiener & Gordon (42) studied the half life of γ-globulin in a patient with agammaglobulinemia with the γ-globulin inhibition technique. He obtained a half life for γ-globulin of approximately 30 to 35 days. The total amount of γ-globulin in an adult is about 55 gm. The rate of γ-globulin production necessary to maintain homeostasis was estimated to be approximately 16 mg. per kg. of body weight per day.

Two cases of acquired idiopathic hypogammaglobulinemia associated

with splenomegaly and hemolytic anemia, caused by hypersplenism, have been reported by Prasad *et al.* (43). Splenomegaly and hypersplenism appear to be the result of reticulum cell hyperplasia caused by the lack of γ-globulin and resultant repeated infections. Because of the obvious therapeutic implications, it is important to recognize this syndrome; serum γ-globulin levels should be determined in cases with unexplained hepatosplenomegaly, hypersplenism, and hemolytic anemia. Good *et al.* (44) studied three patients who had agammaglobulinemia together with an illness indistinguishable from rheumatoid arthritis. The implications of these observations on theories concerning the etiology of rheumatoid arthritis and other fibrinoid diseases are discussed.

Schmidt & Wildhurt (45) studied 310 cases of liver cirrhosis and found 35 cases with a total serum protein of more than 8.5 gm. per cent. In all cases the diagnosis was verified by liver puncture. On paper electrophoresis, the γ-globulin may show a picture very similar to myeloma; consequently liver biopsy might be valuable in cases of suspected "plasmocytoma." Leonhardt (46) found that hypergammaglobulinemia and systemic lupus erythematosus were strikingly frequent in a family of 14 siblings: three of them had the latter disease with high γ-globulin values, and one had a decidedly pathological increase of γ-globulin without other symptoms of active disease. Four other siblings had distinctly pathological increases of γ-globulin but were clinically healthy. The remaining six had normal or slightly increased γ-globulin values and were on the whole healthy. It is suggested that hypergammaglobulinemia may precede other symptoms of the disease.

Laurell & Nilsson (47) found an anticoagulant with antithromboplastin effect in two patients who had hypergammaglobulinemia and hemorrhagic diathesis and whose sera gave false biologic reactions for syphilis. After electrophoretic separation of the serum proteins, both Wassermann reagin and antithromboplastin were localized in the same region as the γ-globulin. The human γ-globulin could be resolved into four components by chromatography on diethylaminoethyl cellulose (48). The reagin content of serum from allergic subjects was almost completely confined to one of these components, which contained about 10 per cent of the total γ-globulin.

Macroglobulins.—Sehon *et al.* (49) investigated sera from four cases of macroglobulinemia by means of zone (paper and starch) and free electrophoresis, ultracentrifugation, and immunologic methods. On free electrophoresis, the macroglobulin fraction of three sera gave rise to single symmetrical peaks with the mobility of a slow-moving γ-globulin, while the macroglobulin fraction of the fourth serum was resolved into three peaks with mobilities of α_2-, β-, and γ-globulins. The macroglobulin contained material rich in carbohydrate. When studied in the ultracentrifuge, the macroglobulin fractions were found to be heterogeneous, with S values of 10.6 to 28.8. The immunologic methods indicated the presence of antigenically specific material in the macroglobulin fraction; this material was absent from normal serum.

Myeloma proteins.—Berson & Yalow (50) studied the serum proteins of a patient with a high serum concentration of myeloma γ-globulin but without Bence-Jones proteinuria. The serum proteins of this patient were labelled with I^{131} and administered intravenously to this subject as well as to another patient who manifested marked Bence-Jones proteinuria but no anomalous serum proteins. The daily production of serum myeloma protein in the donor subject was about two and one half times as great as that of serum albumin in normal subjects. The excretion of protein-bound radioactivity in the recipient subject would have been insufficient to account for the Bence-Jones proteinuria, had the latter been derived from serum myeloma protein. Berson & Yalow suggest that the serum myeloma proteins are not precursors of the Bence-Jones protein, but do not exclude the possibility that Bence-Jones protein is a precursor or abortive product of serum myeloma globulin synthesis.

The interrelationship between serum proteins and urine proteins has also been studied by Osserman *et al.* (51). In a patient with multiple myeloma, the rates of incorporation and degradation of serum albumin, and of the abnormal serum and urine proteins were followed, with orally administered N^{15}-L-aspartic acid as the biosynthetic label. The turnover rate of urine proteins was extremely rapid, with a half life of 1.5 days; the myeloma serum globulin was found to have a half life of 21 days. The maximum isotope concentration in the urine proteins preceded and exceeded the maximum isotope concentration in the serum proteins. The authors suggest that the abnormal serum and urine proteins in myeloma patients are separate constituents, independently elaborated. Putnam & Miyake (52) have made an extensive study of purified Bence-Jones proteins from 14 patients with multiple myeloma, by ultracentrifugal, electrophoretic, amino end group, and immunological analyses. The proteins could be classified into two antigenic types according to the absence or presence of N-terminal aspartic acid. In the former group five proteins lacked detectable N-terminal groups, whereas other specimens had N-terminal tyrosine, isoleucine, etc. The results suggest that Bence-Jones proteins are individually specific, thus raising a question as to their origin and possible metabolic function.

By means of starch gel electrophoresis, new information has been obtained on the serum proteins in multiple myeloma and on the heterogeneity of Bence-Jones proteins (53). Silberman (54) has used starch gel electrophoresis for the differentiation of multiple myelomatosis from macroglobulinemia.

DISEASES IN LIPIDE METABOLISM

Serum lipides.—Atherosclerosis in man is believed to be a consequence of some disorder of lipide metabolism, but attempts to characterize it by measuring the serum lipides have met great difficulties. Lawry *et al.* (55) measured the serum lipides of 2405 human subjects. Of these, 1368 were active, healthy adults of various ages; 273 were men with myocardial infarc-

tion; 141 were men with definite angina pectoris but without detectable infarction; and 23 were women with myocardial infarction. The most notable result was the large variability in the serum levels of the lipide fractions, S_f12 to 20 and S_f20 to 100, in adults of similar age, sex, and clinical status. Among healthy persons under age 50, men had higher levels of all lipides than women of the same age. Those with established myocardial infarction had higher levels of cholesterol and lipoproteins than age-matched men or women without obvious disease. This supports the belief that clinical manifestations of atherosclerosis are associated with disordered lipide metabolism. The levels in men with angina pectoris were intermediate between those of healthy men and men with myocardial infarction. The authors conclude that the small size and great variability of these differences in serum lipide levels between healthy men and women and those with angina pectoris or myocardial infarction prevent efficient application of serum cholesterol and lipoprotein levels to the clinical prediction of coronary heart disease.

Taylor *et al.* (56) increased the level of daily physical activity of nine healthy students in order to observe the effect on serum cholesterol concentration. No significant effect was found.

Two excellent reviews (57, 58) summarize recent knowledge of the influence of dietary fat on the serum lipides.

Experiments in a number of laboratories have shown clearly that isocaloric exchange of different fats in the diet produces an array of serum lipide changes which seems to be related to the degree of unsaturation of the fat (59 to 66). The experiments have shown that some dietary fats produce higher serum cholesterol and phospholipide levels than others but that the triglycerides in the serum are relatively constant and do not vary in parallel with the other lipide groups. The highest levels of cholesterol are found when butter and coconut oil are fed as sole dietary fats; intermediate levels are produced by palm oil, lard, cocoa butter, and olive oil; the lowest levels follow the feeding of peanut, cotton seed, corn, and sunflower oils (65). These changes are produced without alteration in the ratio of free to total cholesterol. According to Ahrens *et al.* (65), these results are best correlated with the mean degree of saturation of the fat (as measured by its iodine value).

A mechanism by which the ingestion of unsaturated fatty acids lowers the serum cholesterol has been suggested by Hellman *et al.* (67). A patient whose serum cholesterol level was 900 mg. per 100 ml. serum on an *ad libitum* diet was given cholesterol-4-C[14] in order to label the body pools of readily exchangeable cholesterol. A balance study was conducted in order to find whether or not the excretion of the body's readily exchangeable cholesterol into the feces was altered by the feeding of different dietary fats. Preliminary results indicated that when the serum cholesterol concentration rose after butter feeding the fecal sterols decreased by almost the same amount; when serum cholesterol fell on corn oil feedings, the fecal sterols increased by almost the same amount. Gordon *et al.* (62) and Bronte-Stewart

et al. (68) found that a fall in the serum cholesterol level attributable to changes in the dietary fat was accompanied by an increase of bile acids in the feces. Their results are thus compatible with those of the balance study with labelled sterol (67).

An effect on thyroid activity after feeding a formula diet containing corn oil and with a low iodine content was obtained by Hodges & Evans (69). The protein-bound iodine values were doubled between the fourth and sixth week of this diet. At the same time, the uptake of radioactive iodine began to rise (the same effect was noticed by Ahrens *et al.* (65)). The subjects remained clinically euthyroid during the whole study. There was a prompt decrease in the serum cholesterol. The effect described does not seem to be caused by a simple iodine deficiency, but it is difficult to find a satisfactory explanation. Further confirmation and extension of these studies are needed.

In this connection it is well worth mentioning that dietary protein may be one determinant of serum lipide concentration in man. Dietary protein may affect serum lipide levels in different ways. If there is a deficiency of labile methyl groups in the diet of rats, hypocholesteremia develops as the liver accumulates fat, even when the diet also contains cholesterol (70, 71). This hypocholesteremia is not affected by type or quantity of dietary fat (72, 73). The feeding of protein deficient in sulfur (i.e., alpha-protein of soybeans) leads to hypercholesteremia in cholesterol-fed monkeys (74). Portman & Mann (75) showed that this type of sulfur deficiency inhibits the production of taurine and thence of taurine-conjugated bile acids. Thus, when the conversion of cholesterol to taurine-conjugated bile acids is limited, cholesterol accumulates in plasma. The "protective" effect of dietary protein against hypercholesteremia in cholesterol-fed chickens, reported by Nishida *et al.* (76) and Moyer *et al.* (77), may be attributable to increased sulfur requirements for conversion of cholesterol to conjugated bile acids. A similar effect may explain the hypercholesteremia in cholesterol-fed rats on low-protein, high-choline diets (78). Olson *et al.* (72, 73) recently reported well-controlled experiments in man which dealt with dietary protein and serum cholesterol levels. They were able to produce hypocholesteremia with a low-protein moderate-fat diet. The experiments described demonstrate that it is important to clarify the relationship of dietary proteins to serum lipide levels.

The observations that atheromatous plaques are predominantly lipide in composition have encouraged many attempts to correlate the incidence of coronary artery disease with abnormalities of lipide metabolism. James *et al.* (79) used gas-liquid chromatography (80) to analyze the fatty acids from C_6 to C_{20} in the blood of 12 patients with coronary heart disease and in 12 controls matched for sex and age. The fatty acids in phospholipides and nonphospholipides showed no significant differences.

Metabolism of unesterified fatty acids of plasma.—The small quantity of unesterified fatty acids found in human plasma as an albumin-bound com-

plex has been identified by independent work in different laboratories (81 to 84) as a major transport form of fatty acids; this accounts for the bulk of the movement of fatty acids from adipose tissue depots to tissues that require fat as an energy-yielding substrate.

The hormonal control of unesterified fatty acids has been studied by several workers (85 to 89). Gordon (87) demonstrated that an injection of insulin and glucose abolishes the usual arteriovenous increment in the concentration of unesterified fatty acids in the blood from the saphenous vein which drains adipose tissue. Bierman *et al.* (85) compared the clearance of C^{14}-labelled palmitic acid before and after the administration of insulin. The results from these investigations suggest that an increased metabolism of glucose diminishes the output of fatty acids from tissue stores and that insulin causes a fall in the concentration of unesterified fatty acids in plasma by inhibiting the release of fatty acids from tissue stores. The system in plasma thus seems to be abnormal in diabetes. Further study is needed of the relationship between the chronic disturbances of lipide metabolism in diabetes and unesterified fatty acid levels.

Recent work has indicated that clearing factor is a lipolytic enzyme. Korn (90, 91) has described this enzyme as a heparin-activated lipoprotein lipase. By incubating lipoprotein lipase with bacterial heparinase (92), he has recently found evidence for the presence of a heparin-like mucopolysaccharide as an integral part of the enzyme. An interesting observation was made by Klein & Lever (93), who found that serum from patients with primary and secondary hyperlipemia inhibited the clearing activity present in normal serum after intravenous administration of heparin.

SEROTONIN METABOLISM

Many new papers on malignant carcinoid have appeared in the last years. Udenfriend *et al.* (94) have made a thorough investigation of tryptophan metabolism in several patients with this disease. Since the serotonin (5-hydroxy tryptamine) pathway of tryptophan metabolism is here turned from a minor into a major route for the metabolism of tryptophan, it could be suspected that a secondary tryptophan deficiency with decreased formation of other products, such as protein and niacin, could result. In accordance with this hypothesis the urinary excretion of N'-methylnicotinamide was low in patients with a high excretion of 5-hydroxyindoleacetic acid. The fasting serum tryptophan level was also below normal. The tumor pool of serotonin, its turnover rate, and the tumor mass were calculated in one patient.

Pernow & Waldenström (95) report on a study of 33 cases of malignant carcinoid. Determinations of serotonin and 5-hydroxyindole acetic acid were made on the patients: Serotonin values were high in either blood or urine or in both in 17 of 18 cases, and abnormally high 5-hydroxyindole acetic acid values were obtained in 19 of 20 cases. In 11 cases in which the tumor had been removed, probably *in toto,* the values for serotonin and 5-hydroxy-

indole acetic acid were normal or only slightly increased. In eight cases the urinary excretion of histamine was studied as well. An increased excretion, with figures ranging from 27 to 6800 µg. per 24 hr., was found in seven cases. The reason for the high excretion of histamine in some cases is not clear. These authors think that either the tumor cells produce both serotonin and histamine or the circulating serotonin leads to a secondary release of histamine. The latter possibility would be in conformity with the results of Feldberg & Smith (96), who showed that serotonin can release histamine from living cells.

In connection with the findings of a high histamine excretion in certain cases of malignant carcinoid the work of Hanson (97) is of interest. Hanson found in these cases not only a high excretion of histamine but also an increased excretion of imidazole acetic acid—a major product of the oxidation of histamine *in vitro*. An interesting atypical carcinoid tumor has been described by Sandler & Snow (98). The patient—a man aged 45—had a tumor in his ventricle and large hepatic metastases. He had flushing attacks precipitated by food and alcohol. His excretion of 5-hydroxyindole acetic acid was small, but his urine contained a fair amount of serotonin and a large amount of 5-hydroxytryptophan. Histamine was also present in large amounts. Smith *et al.* (99) have described a similar case in which the tumor tissue resembled that of a carcinoid, but no typical argentaffin granules were found. These authors put forward the hypothesis that the argyrophil cells found in their patient form 5-hydroxytryptophan, whereas the argentaffin cells with typical granules contain 5-hydroxytryptophan decarboxylase and convert the substrate into serotonin. They also think that the patient described by Waldenström *et al.* (100), who excreted large amounts of serotonin and histamine (but wherein no determinations of 5-hydroxytryptophan were made) might belong to the same group.

An interesting connection has been found between serotonin metabolism and Fölling's disease. Although brain tissue contains serotonin (101, 102, 103), the findings in patients with malignant carcinoid and with elevated blood serotonin values (but without cerebral symptoms) indicate, however, that the brain-blood barrier is impermeable to serotonin. Udenfriend *et al.* (104) have found that its precursor, 5-hydroxytryptophan, readily penetrates into almost all body tissues, including the brain, and is converted to serotonin in those organs containing 5-hydroxytryptophan decarboxylase. The same authors showed, as early as 1956 (105), that administration of 5-hydroxtryptophan led to an increase in brain serotonin and to marked central disturbances, with effects resembling those seen with indole hallucinogenic drugs or after the administration of reserpine or iproniazid (1-isonicotinyl-2-isopropylhydrazin) (see also 106). Now Pare *et al.* (107) have found that the levels of both serotonin in blood and 5-hydroxyindole acetic acid in urine were below normal in children with phenylketonuria. The authors think that this secondary deficiency in tryptophan metabolism might be the real cause of the mental symptoms of these patients. The same authors

have started a campaign (108) to treat suitable phenylketonuria patients with 5-hydroxytryptophan. It will be very interesting to learn the result of this trial.

Anderson *et al.* (109) accidentally observed a 24-fold increase in the 24-hr. excretion of 5-hydroxyindole acetic acid after giving a banana, as reward for satisfactory performance, to a monkey maintained on chow diet. They found that iproniazid, when administered to monkeys after four days of banana feeding, led to a prompt decrease in the high excretion previously noticed; they concluded that bananas must contain a precursor of 5-hydroxyindole acetic acid from which this acid could be formed by oxidative deamination. That this conclusion was correct and that the precursor was serotonin were shown by Waalkes *et al.* (110), who were able to demonstrate that banana pulp contains as much as 3.7 mg. of serotonin per banana, or about 28 μg. per gm. pulp. Incidentally, bananas are also rich in norepinephrine and dopamine. The presence of these potent physiologic agents in a food as widely used as the banana is of course of clinical interest. Whether the oral administration of these amines through banana feeding can have effects on the gastrointestinal tract or the cardiovascular system remains to be determined. One might also speculate whether some of the reported therapeutic effects of bananas (in celiac disease, peptic ulcer, constipation, and so forth) may be traceable to the presence of these amines. Of immediate clinical significance is the fact that ingestion of bananas may lead to erroneous diagnoses of malignant carcinoid and pheochromocytoma by producing an increased urinary excretion of serotonin and norepinephrine and their metabolites. Whether other fruits which, like bananas, darken on exposure to air also contain amines in similar quantities has still to be determined.

METABOLISM OF EPINEPHRINE AND NOREPINEPHRINE AND THE DIAGNOSIS OF PHEOCHROMOCYTOMA

In 1956 Armstrong *et al.* (111) made an extensive study of the phenolic acids of human urine. Among the substances found, two should prove to be of great theoretical and practical interest: homovanillic or 3-methoxy-4-hydroxyphenylacetic acid and 3-methoxy-4-hydroxymandelic acid. Homovanillic acid was later (112) shown to be formed by methylation of homoprotocatechuic acid, and Armstrong & McMillan (113) therefore thought it possible that a similar methylation of the 3,4-dihydroxy group of norepinephrine might lead to the 3-methoxy-4-hydroxymandelic acid. This hypothesis was supported by the following facts: most adults excrete about 1.5 to 3 mg. of the latter substance per gm. creatinine, corresponding to 2 to 4 mg./day; an increased amount was excreted by two patients when given intravenous norepinephrine for support of blood pressure, and 30 per cent of the administered norepinephrine could be accounted for as extra 3-methoxy-4-hydroxymandelic acid; two patients with pheochromocytomas excreted, respectively, 12 and 90 mg. per gm. creatinine, preoperatively, and 1.5 and 2.7 mg., postoperatively. The stability of 3-methoxy-4-hydroxy-

mandelic acid, the amount present, and relative ease of estimation makes its determination, rather than of catechol amines, preferable for the detection of pheochromocytomas. Since it has been shown that radioactive norepinephrine and epinephrine give the same pattern of radioactive urinary metabolites (114), it is likely that both amines contribute to the 3-methoxy-4-hydroxymandelic acid in urine. But under usual conditions most of the substance excreted probably arises from norepinephrine.

The hypothesis of Armstrong *et al.* has found ample support in the work of Axelrod and his co-workers (115 to 118). He has found a specific enzymatic system for this methylation, which is present in many organs, including liver and brain. He has also found large amounts of methoxy-epinephrine and methoxynorepinephrine in the urine after injection of epinephrine and norepinephrine into rats. Large amounts of normetanephrine were found in the urine from human subjects with pheochromocytomas. When suitable methods have been developed, the estimation of these substances and of 3-methoxy-4-hydroxymandelic acid in urine will probably be of great help in the diagnosis of diseases of epinephrine and norepinephrine metabolism (see also 119). From a theoretical point of view, it will be of interest to know whether the methylated compounds are physiologically active.

Concerning the origin of homovanillic acid, the following facts are now known: Booth *et al.* (120, 121) found that this substance and increased amounts of meta-hydroxyphenylacetic acid were excreted after the ingestion of homoprotocatechuic acid and that all three acids were excreted after the ingestion of DOPA. Shaw, McMillan & Armstrong (112) studied the metabolism of DOPA after the ingestion of 500 mg. of L-DOPA; thirty-five per cent appeared as homovanillic acid and 45 per cent as homoprotocatechuic acid in the urine. Another substance which yields homovanillic acid has also been detected by Booth *et al.* (120, 121). By analyzing rabbit urine after the oral administration of rutin, quercetin, or 3,4-dihydroxyphenylacetic acid, these authors found an increased excretion of homovanillic acid.

URTICARIA PIGMENTOSA

Urticaria pigmentosa is a disease in which the skin and other tissues are very rich in mast cells. Mast cells contain heparin (122), histamine (123), and serotonin (124, 125). The patients suffering from this disease have, therefore, abnormally large stores of histamine and serotonin which can be released, for instance, in allergic reactions and thereby cause severe shock. The most typical symptom is a flush, somewhat resembling that seen in malignant carcinoid, followed by excretion of histamine, serotonin, and their metabolites [cf. malignant carcinoid (126, 127)]. Of great interest in this connection is the work of Schayer *et al.*, who have shown that histamine is to a large extent methylated before oxidative deamination and that the main urinary excretion product in man seems to be 1,4-methylimidazole acetic acid (128 to 131).

The Biochemistry of Gout

Thanks to the work of two groups, Weissmann's at the Mount Sinai Hospital and Wyngaarden's at the National Institutes of Health, many problems concerning the hyperuricemia in primary and secondary gout have been clarified. Weissmann and his group have studied the purine bases of endogenous origin in the urine of human beings in health and disease. Among the substances found, 6-succinoaminopurine and 8-hydroxy-7-methylguanine are of special interest (132, 133, 134). The phosphoribosyl derivative of 6-succinoaminopurine adenylosuccinic acid is presumed to represent the immediate precursor of adenylic acid in nucleic acid (135). Weissmann & Gutman (134) now think that the occurrence of free succinoaminopurine in human urine results from the presence at the site of synthesis or elsewhere, of an enzyme or enzymes capable of splitting ribose phosphate from the adenylosuccinic acid. Free succinoaminopurine thus liberated is then eventually excreted in the urine. If this is so, it would be reasonable to expect that the amount of this substance in urine would parallel the rate of nucleic acid synthesis. Consistent with such a relationship is the finding (136) that the urinary excretion of succinoaminopurine is increased in polycythemia vera. The origin of 8-hydroxy-7-methylguanine is not so clear, but Weissmann & Gutman think that it is derived from nucleotide precursors of nucleic acid guanine. If so, the amount of this substance should reflect also the rate of synthesis of nucleic acids. In agreement with such a view is the greatly increased excretion of 8-hydroxy-7-methylguanine, also found in polycythemia vera (136).

On the basis of the two before-mentioned assumptions, Weissmann & Gutman put forward an interesting hypothesis to explain the metabolic error in primary gout. The available data (137) indicate a normal excretion of 8-hydroxy-7-methylguanine in interval gout and a large increase during the acute attack. On the other hand, the urinary output of succinoaminopurine is larger than normal in the quiescent phase of the disease but declines during the acute phase. This suggests to the authors that in gout there may be an imbalance between the relative amounts of the guanine and adenine precursors of nucleic acid produced. The precursors produced in excess because of this imbalance would be degraded and should eventually appear as uric acid. They might in this way contribute to the overproduction of uric acid observed in some individuals with gout. It is, however, not yet quite clear whether such an overproduction really exists. As early as 1953 Stetten et al. (138) had found that some gouty individuals showed a more rapid incorporation of N^{15}-glycine into urinary uric acid than normal persons. The incorporation was so unusually rapid in some gouty subjects that it was necessary to postulate the existence of a shunt whereby dietary glycine nitrogen could enter the purine nucleus of uric acid more promptly than in normal man, presumably without the intervention of the nucleic acid purines (139), the turnover of which appeared to be too slow to account for these results (see also 140).

In several articles Wyngaarden *et al.* emphasized the overproduction of uric acid in primary gout (141, 142, 143). They found a consistently excessive incorporation of glycine-1-C^{14} in a gouty patient. But in a recent paper (144) Wyngaarden has described two gouty subjects with normal incorporation. He is now doubtful whether the incorporation test with labelled glycine gives any reliable information concerning the production rate. Similar conclusions are drawn by Seegmiller *et al.* (145).

Also of great interest is a paper of Weissmann *et al.* (146), in which the biosynthesis of uric acid from glycine-N^{15} in primary and secondary polycythemia is reported. It was demonstrated by the magnitude of cumulative isotope labelling that the hypouricemia in some cases of polycythemia is attributable to overproduction of uric acid, as has long been suspected. The incorporation in polycythemia vera was slow with a peak at the end of the second week after the ingestion. The time relationships were consistent with conversion of glycine into the purine components of intracellular nucleic acids, and subsequent transformation into uric acid. The rate curves of glycine-N^{15} incorporation into urinary uric acid in secondary polycythemia differed somewhat from those found in primary polycythemia vera, at least in the late phases examined. The findings (in 136, 144) might indicate that primary and secondary gout may differ in uric acid metabolism. In some cases, at least of primary gout, there seems to be a metabolic shunt for the production of uric acid without the intervention of intracellular nucleic acids. In secondary gout, on the contrary, the overproduction of uric acid is caused by the increased production of intracellular nucleic acids. A good review (146) of the intermediary purine metabolism and the metabolic defects of gout by Wyngaarden covers the literature up to September 1956.

IRON METABOLISM IN DISEASE

Moore has published a review of his excellent work on iron absorption (147). In normal adults, 5 to 10 per cent of the food iron is absorbed. As an ordinary diet contains 12 to 15 mg. iron, the daily retention amounts to about 1 mg. The amount of nonhemoglobin iron lost daily lies between 0.5 to 1 mg. Nutritional iron deficiency can therefore easily occur in growing children and menstruating women. In men and postmenopausal women, dietary iron deficiency is very unlikely to occur.

Finch *et al.* (148 to 152) are continuing their work on iron metabolism. Of special interest are their studies of transferrin iron turnover with isotope techniques under various conditions. The factor which seems to have the greatest influence on this is the activity of the erythropoietic bone marrow. An overactivity of the marrow might increase the turnover from three to six times. Increased hemolysis produced by injection of stored red blood corpuscles increased the serum iron up to the saturation limit but did not significantly increase the turnover. It is, therefore, clear that the iron released in the phagocytes must stay in a slow-moving pool if it exceeds the

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amount that can be utilized by the marrow. Under the experimental conditions, the greatest amount was found in the liver. Some cases of hemochromatosis were also studied. They showed a moderate (about 50 per cent) increase in turnover.

Green & co-workers (153, 154) have discovered an interesting mechanism which promotes the exchange of iron between ferritin and transferrin. They have noticed that reduced xanthine oxidase reduces the ferric iron of ferritin and that this iron can be picked up by transferrin. Intravenous injection of xanthine or hypoxanthine leads to an elevated plasma iron in rabbits and dogs, and the authors conclude that the release of iron from the ferritin stores of the liver might be limited by the level of xanthine and hypoxanthine in the hepatic cells. If a similar mechanism should exist in the intestinal mucosa cells this finding might be of great practical interest.

Three publications which contain interesting observations on iron metabolism in hemochromatosis should be mentioned (155, 156, 157). An excellent monograph by Dreyfus & Schapira (158) on the biology of iron has recently appeared.

COPPER METABOLISM AND DISEASE

The real significance of the low ceruloplasmin levels in serum in Wilson's disease is still not clear. Jensen & Kamin (159) studied the fate of Cu^{64} in normal subjects, in patients with Wilson's disease, and in patients with Laennec's cirrhosis. As reported earlier by Bearn & Kunkel (160), they found in normals an initial peak in loosely bound serum copper followed by a peak in ceruloplasmin copper about 40 hr. after the ingestion. In patients with Wilson's disease this secondary peak was lower than normal, or it was absent, whereas it was higher than normal in patients with Laennec's cirrhosis (see also 161). Scheinberg & Morrell (162, 163, 164) have used Cu^{64} to study the exchange between ceruloplasmin copper and ionic copper. They made the interesting observation that such an exchange took place only when ceruloplasmin was reduced by ascorbic acid. The experiments were performed at pH 5.8, and at this pH about half the copper atoms of ceruloplasmin seemed to be exchangeable. This finding is of theoretical interest, especially as Laurell in this laboratory showed several years ago that ascorbic acid only reduces half of the copper atoms of the ceruloplasmin molecule, which is at this point completely decolorized. In effect, the authors think that they have found a support for the hypothesis that ceruloplasmin is a vehicle for the transport of copper in blood. At the physiological pH of plasma the copper of ceruloplasmin will, however, be much more firmly bound, and it seems very doubtful that any measurable exchange will take place *in vivo*.

Recently there has been a great interest in ceruloplasmin in connection with schizophrenia. As far back as 1941 Heilmeyer *et al.* (165), reported the finding of elevated serum copper values in some cases of schizophrenia. It should be mentioned that this finding was not supported by an early

unpublished study in this laboratory. In recent years the interest in ceruloplasmin in connection with schizophrenia has been revived through the work of Heath *et al.* (166). This team reports on the preparation of a substance, which they call taraxein, from the serum of schizophrenic subjects. When injected into men and monkeys, this substance is supposed to cause mental changes similar to those seen in schizophrenia. When preparing ceruloplasmin from the blood of schizophrenic subjects, according to the method of Holmberg & Laurell, Heath *et al.* noticed that in one of the steps they got a blue color in the supernatant, which was not obtained with the blood from normal subjects. They suspect, apparently, that this might be attributable to an abnormal ceruloplasmin, and it is this fraction which they have purified further and now call taraxein. The results of Heath and collaborators have been questioned and still await confirmation.

Åkerfeldt (167) has attacked the problem from another angle. When N,N-dimethyl-*p*-phenylenediamine is added to serum, it is attacked by ceruloplasmin, and, after a lag period, a red color develops. Åkerfeldt noticed that the oxidation was more rapid and the lag period shorter than normal in the serum from patients in a mental hospital. Later he found that the main reason for this difference was the low content of ascorbic acid in the blood of these patients. Several authors (see, for instance, 168, 169) have conclusively shown that neither the content of ceruloplasmin nor the intensity of the oxidase reaction differs significantly from the normal in schizophrenics.

Still another approach to the problem comes from Mårtens *et al.* (170). These authors claim that the intravenous injection of large amounts of ceruloplasmin to schizophrenics causes a more or less complete disappearance of the mental symptoms. As the injections of their preparation produced very marked vasomotor disturbances, it remains to be seen whether the effect noticed is specific. The author of this review (Holmberg) is still far from convinced that quantitative or qualitative alterations in ceruloplasmin have anything to do with the development of schizophrenia.

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THE BIOCHEMISTRY OF GENETIC FACTORS[1,2]

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INTRODUCTION

The genetic material comprises those parts of the cell which are responsible for the maintenance and transmission of hereditary characteristics, and genetic factors can be distinguished within the genetic material through their capacity for individual mutation. Two main approaches to the present subject may be distinguished. One starts with the genetic material itself (in so far as it can be identified), and deals with the biochemical mechanisms of its replication and its control of other cell processes. The second consists of the study of the biochemical effects of mutations and seeks, by a combination of biochemical and genetical methods, to analyse the genetic material into units of specialised function. This review will attempt to cover both aspects.

GENETIC TERMINOLOGY

Although some genetic differences in moulds, green plants, and animals appear to have their basis in the cytoplasm, most are capable of being associated with particular loci of the chromosomes. All the available evidence (1, 2) indicates that inherited variation in those bacteria which have been studied also has a chromosomal basis. "Chromosome," in this sense, means a linear structure along the length of which genetic differences can be located by breeding experiments and does not necessarily imply any close structural similarity btween bacterial chromosomes and the enormously larger chromosomes of higher plants and animals. The term "gene" has lately become rather ambiguous, and it is now recognised that several kinds of chromosomal unit may be distinguished (3). In this review the term "locus" refers to a short chromosome region of specific function; by "alleles" are meant alternative inherited states of a given locus.

NATURE AND MODE OF ACTION OF THE GENETIC MATERIAL

Genetic function of nucleic acids.—Recent investigations have been dominated by the hypothesis that the genetic material is deoxyribose nucleic acid (DNA). While many facts tend to support this hypothesis, it is by no means established as generally true. Little critical evidence is available from higher organisms. It has been commonly supposed that the genetic material is DNA on the ground that DNA is a major component of the chromosomes

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[1] The survey of the literature pertaining to this review was completed in September 1959.

[2] The following abbreviations are used: DNA for deoxyribonucleic acid; RNA for ribonucleic acid.

and, generally speaking, uniquely associated with them. Furthermore, the chromosome set of a given species has been generally found to contain a constant and characteristic amount of DNA which doubles more or less abruptly at the time of chromosome division. This type of behaviour may also be characteristic of some chromosomal proteins, however. Recent reviews of the cytochemical approach to the problem have been made by Alfert (4) and Taylor (5). The apparent absence of DNA from certain echinoderm eggs has been cited against the identification of DNA with the genetic material (6), but the observation is not generally accepted [see discussion following Ris's paper (7)].

More evidence is available from microorganisms, the most convincing being that from the study of the transforming principles of *Pneumococcus* and *Hemophilus influenzae* (8, 9, 10), which have been identified with near certainty as DNA. The proportionality, found both in *Pneumococcus* (11) and in *Hemophilus* (9), between transformation frequency and the amount of DNA taken up by the cells implies that one molecule of DNA can transform a cell; in both organisms the uptake by a cell suspension of fewer than 100 molecules of DNA (mol. wt. 15×10^6) of the most active preparations is found to suffice for the transformation of one cell with respect to a given single mutational difference. In general it has been found that determinants of different characters are acquired independently of one another during transformation, and in one case (12) Ephrussi-Taylor has shown that two determinants are associated with different DNA molecules. Hotchkiss and co-workers (13, 14) have presented preliminary evidence from *Pneumococcus* that the DNA determinant of a given character (in the cases studied, sulphonamide resistance and ability to hydrolyse maltose, both thought to be based on single enzymes) can be altered by mutation in different regions, and the linked mutational sites are capable of being separated and recombined during transformation. Linkage between the determinants of different characters is now well known (13), so it seems that a single DNA particle can carry determinants of more than one character and also display complex structure within a single determinant. The transfer of the information corresponding to a single mutational difference need not require a complete DNA molecule of the size observed in transforming preparations. For example, a study of the decrease in transforming activity as a result of the scission of DNA molecules by ultrasound (15) has indicated that a piece of DNA equivalent to a mol. wt. of 1×10^6 is sufficient in each of three different transforming systems in *Pneumococcus*. If different DNA molecules differ genetically, one might hope that they would differ chemically and physically also. Although it has been possible to fractionate transforming DNA by chromatographic methods (16), little progress has so far been made in the separation of different transforming activities into different fractions. However, differences have been shown between determinants of different characters in their stability under various treatments, including ultraviolet irradiation, heat, and nitrogen mustard. The relative stabilities of dif-

ferent determinants were not greatly altered by passage through different bacterial strains (17). Recently the study of transformation by DNA preparations has been extended to another bacterial species, *Xanthomonas phaseoli* (18, 19). In *Escherichia coli*, Jacob & Wollman (20) have studied the decline in the capacity of cells labelled with P^{32} to transfer genetic markers by conjugation. They have shown that the probability of a given marker's losing its transferability as a result of P^{32} decay is nearly proportional to its genetic distance from the point on the chromosome which is injected first during genetic transfer; this work goes some way toward establishing a direct relationship between amount of DNA and genetically measured chromosome length.

In bacteriophage there are strong indications that DNA is responsible for genetic continuity. The main items of evidence are that virtually all the DNA but very little of the protein of a bacteriophage enters the host cell on infection (21, 22) and that P^{32}-labelled phage is highly vulnerable to P^{32} decay (23). On the other hand, Stent and others have shown that the T-even phages reach a stage of growth within the host cell during which they become highly resistant both to P^{32} decay and to ultraviolet irradiation. This and other evidence from bacteriophage studies reviewed by Stent (23) has led him to suggest that DNA does not replicate directly, but rather acts as a template for the formation of ribonucleoprotein (which might well be more resistant than DNA to P^{32} decay and ultraviolet light) which then serves, in turn, as a template for production of more DNA. Stent proposes a mechanism through which this might occur, but its feasibility has yet to be checked by accurate model building. If the suggestion is correct, it means that there is no single type of molecule which can be considered *the* genetic material. In most organisms studied it is almost certainly the DNA which is responsible for transmitting the genetic information from one sexual generation to the next, but, on Stent's hypothesis, this role could be filled by RNA or ribonucleoprotein in some forms. Recent work on tobacco mosaic virus has afforded clear evidence for the genetic role of RNA in plant viruses. The decisive observations are that apparently protein-free RNA can infect plants and lead to the production of complete virus (24), and that virus particles reconstituted from RNA isolated from one strain and protein from a different strain have been found to propagate virus resembling that from which the RNA had been derived (25).

Mutation.—Various treatments which are expected to interfere with DNA synthesis have been shown to cause mutations. Both 5-bromouracil (26), which can become incorporated into DNA in place of thymine, and 2-aminopurine, an adenine analogue (27), are effective mutagens for bacteriophage T4. The sites within a genetic locus which are most susceptible to these mutagens tend to be different from those which mutate spontaneously with appreciable frequency (26, 27), and the two analogues differ from each other in the mutations which they induce; only one site was found which responded to both (27). Thymine starvation has been reported

to induce mutations in *E. coli* (28). Several studies (e.g., 29, 30) indicate that protein synthesis is necessary for the establishment of ultraviolet-induced mutations in bacteria, and a preliminary report (30) tends to implicate RNA synthesis also.

Mechanism of replication of DNA.—An essential property of genetic material is that it should provide the pattern for its own replication, and one of the attractive features of the structure for DNA proposed by Watson & Crick and now generally accepted (31) is a suggested mechanism (32). The original proposal of Watson & Crick was that, during replication of DNA, the two complementary strands of the duplex molecule were separated and a new complementary strand synthesised alongside each one. The main difficulty has been to explain how the relationally coiled strands are able to separate. Various ideas for overcoming this difficulty are discussed by Delbruck & Stent (33) in whose terminology the Watson-Crick mechanism is a semiconservative one, as opposed to a conservative mechanism in which the whole parental duplex would remain intact, or a dispersive one in which neither strand of the parental duplex would do so. The mechanism recently proposed by Stent (23), referred to in the preceding section, is basically conservative, but could appear dispersive if the DNA were subject to breakdown and resynthesis, as it could be in Stent's scheme. Meselson & Stahl (34) have provided impressive evidence for a semiconservative replication of DNA molecules in *E. coli*. Cultures grown on $N^{15}H_4^+$ as sole nitrogen source were transferred to N^{14} medium and at the same time, and at various times thereafter, cell samples were lysed and the density of their DNA (which was uniform in molecular size) was determined by density-gradient centrifugation, a technique which permitted a clear separation of N^{15}-DNA from N^{14}-DNA. After one cell generation time, the N^{15}-DNA had been entirely replaced by DNA of a density which indicated that 50 per cent of its nitrogen was N^{15}; after two generation times, nearly equal amounts of unlabelled and half-labelled DNA were found. During subsequent divisions half-labelled molecules continued to appear but as a decreasing proportion of the total. This result is exactly what would be expected if the Watson-Crick model for replication were true. It is possible, however, that the two parts of the bipartite DNA molecule which is clearly indicated by this experiment are not the two strands of a double helix but may be double helices in their own right. That this may be so is suggested by the observation that the half-labelled DNA could be dissociated into its labelled and unlabelled components by heat, while salmon sperm DNA did not dissociate under the same conditions.

Kornberg and co-workers (35, 36) have isolated and highly purified an enzyme from *E. coli* which will synthesize DNA from a mixture of the four deoxyribonucleoside triphosphates in the presence of magnesium ion and polymerized DNA. All four triphosphates are necessary for appreciable synthesis, and the addition of the DNA is absolutely essential; the DNA formed by the system has exceeded the amount initially added by more than

tenfold. It should be possible to determine whether the DNA formed inherits any biological specificity from the DNA added to the system and whether the material of the added DNA is distributed among newly-formed molecules in the manner indicated by Meselson & Stahl's *in vivo* study.

A kind of semiconservative replication of bacteriophage DNA is indicated by Levinthal's experiments (37, 38). Using an autoradiographic technique for measuring the P^{32} content of individual particles, he found (a) that after one cycle of infection a few progeny particles each contained a piece of parental DNA corresponding to about 20 per cent of the total DNA of a phage particle, (b) that about 40 per cent of the DNA of a phage particle was dispersed after osmotic shock, and (c) that the 20 per cent pieces of parental DNA formed part of this nondispersible fraction. Levinthal's interpretation is that the nondispersible fraction replicates according to the Watson-Crick scheme, while the rest is dispersed widely among the progeny. Rather similar conclusions have been reached by Stent on the basis of quite different experiments [(33, 39); see also review by Hershey & Burgi (40)]. Experiments designed to show whether genetic markers are transmitted with the semiconservative or with the dispersive part of the DNA have given conflicting results (33, 41).

Using an autoradiographic technique, Taylor and his colleagues obtained evidence that when a bean chromosome labelled in its DNA with tritiated thymidine divided in the absence of further label, the radioactivity was equally distributed between the two daughter chromatids at the first metaphase after labelling, but was segregated into one of each pair of daughter chromatids at the next division (42). Taylor has reported similar findings with another plant, *Bellevalia romana* (43, 44). Plaut & Mazia (45) had earlier claimed a different result with chromosomes of *Crepis capillaris* with the label (in this case C^{14}-thymidine) showing markedly unequal distributions between daughter nuclei at the *first* division after incorporation of the isotope, and this result has recently been reasserted by Plaut (46). The formal similarity between Taylor's results with chromosomes and those of Meselson and Stahl on DNA molecules is remarkable in view of the relatively great size of the plant chromosome. In a paper which includes a useful review of the information on chromosome division, Taylor (5) proposes a structure for the chromatid consisting of a double-stranded protein backbone with DNA molecules attached as side chains, one strand of each DNA duplex being attached to each strand of the backbone. It is not clear what significance is to be attached to the conclusions drawn from electron microscope studies (7, 47) that plant and animal chromosomes may consist of relatively large numbers of strands (as many as 64 or 128 in *Tradescantia*, each about 125 A (47) or 200 A (7) in diameter.

Information transfer and the coding problem.—To judge from the effects of mutations (see next section), the genetic material acts largely, if not entirely, through controlling the synthesis of specific proteins. In contrast to the abundant evidence which now exists for the involvement of RNA in

protein synthesis (48), relatively little information is available to implicate DNA directly in this process (49). Thus, if one attributes a genetic function to DNA, it is natural to look for some link between DNA and RNA which could enable RNA to act as a carrier of information from DNA to protein. An idea much favoured at present (50) is that DNA acts as a template in RNA formation in the nucleus, and that nuclear RNA passes into the cytoplasm (probably into the microsomal particles) where it provides in turn templates for protein synthesis. Stent, indeed (23), has suggested that DNA-mediated RNA synthesis may be an essential part of DNA replication. Despite the attractiveness of the hypothesis, derived, as Crick (50) points out, from the difficulty of formulating any other coherent alternative, the template function of microsomal RNA is at present no more than an assumption, and the biochemical evidence for the nuclear origin of cytoplasmic RNA is controversial. While much evidence on the time course of *in vivo* incorporation of C^{14}-adenine (51) and radioactive phosphate (52) into different RNA fractions is consistent with the idea that the nucleus is the site of primary RNA synthesis, Osawa *et al.* (53), who determined the distribution of P^{32} between the four bases of cytoplasmic RNA and two nuclear RNA fractions of calf thymus, have concluded that not all the cytoplasmic RNA could have been of nuclear origin; in this they agree with the earlier conclusions of Barnum *et al.* for mouse sarcoma (54). Smellie *et al.* (52) concluded that the nuclear precusor hypothesis was more consistent with the data for some tissues than others in the rabbit. The more direct approach made possible by autoradiographic techniques has been utilised by several authors, whose work tends to support a nuclear origin for at least much cytoplasmic RNA. Goldstein & Plaut (55) transplanted a nucleus from a P^{32}-labelled amoeba to an unlabelled one and found that phosphorus passed from the labelled nucleus into cytoplasm but not into the unlabelled nucleus native to the recipient cell. Zalokar (56) stratified cells of *Neurospora* mycelium by centrifugation and followed changes with time in the distribution of radioactivity among the various cell fractions following assimilation of C^{14}-proline or tritiated uridine. He concluded that protein was synthesised in the microsomal fraction but that RNA was synthesised in the nucleus and was transferred to the microsomes. Woods & Taylor (57) reached similar conclusions regarding the nuclear origin and transfer to the cytoplasm of RNA from the study of radioautographs of bean root cells after assimilation of tritiated uridine; these authors identified the nucleolus as the main site of RNA synthesis (or of rapid accumulation after synthesis) within the nucleus.

If the sequence of amino acids in a protein is determined by nucleic acid and if the information carried in the nucleic acid is a function of the sequence of nucleotide bases, one is faced with the problem of how the ordering of 20 amino acids can be specified in a code built with only four different bases. It is clear that at least three bases must be involved in the determination of each amino acid, since a code based on pairs of bases could give only

sixteen different alternatives. If, in a code based on triplets of bases, the triplets overlap, each base being a member of three successive triplets, then a restriction is placed on the number of possible dipeptide sequences in proteins, and Brenner (58) has shown that the number of different amino acid sequences already known is more than is compatible with *any* overlaping triplet code (on the reasonable assumption that the same code applies to all organisms). Crick (50) and Crick and co-workers (59) have investigated the possibilities of a nonoverlapping triplet code in which the polynucleotide chain is functionally divided into trinucleotide segments by the provision that trinucleotide sequences overlapping two segments must not mean anything in the code. They have shown that the construction of such a code is possible and that 20 different amino acids, *but no more,* can be provided for. This is a satisfactory result, provided that amino acids other than Crick's "magic twenty," such as hydroxyproline, can be regarded as substituting for members of the normal set or as derived from them after incorporation.

Both Stent (23) and Zubay (60) have suggested that the base sequence of RNA may be determined by DNA through each RNA base corresponding to one of the four possible *pairs* of bases (adenine-thymine, thymine-adenine, cytosine-guanine, guanine-cytosine) of the DNA double helix. Zubay has proposed a specific coding on the basis of observed correlations between the base compositions of DNA and nuclear RNA in four different materials; unfortunately Zubay's code is the exact opposite of that suggested by Stent on stereochemical grounds. The correlation reported by Belozersky & Spirin (61) between the base compositions of DNA and total RNA in various species of bacteria tends to support Stent's code.

BIOCHEMICAL EFFECTS OF ALLELIC DIFFERENCES

Effects on enzyme formation.—Most nutritional mutants of microorganisms can be made to grow normally by supplying a single substance, a fact which was for many years the main support for the "one gene–one enzyme" hypothesis. Although some disillusionment was caused by demonstrations that nutritional requirements were not always easily explicable in terms of effects on enzymes (62, 63), several cases are now known in which the "one enzyme" explanation appears to be justified. A list of such cases is shown in Table I. Also included in the table are several types of genetic defect in man; these may be analogous to the defects in biochemical mutants of microorganisms since they behave genetically as if resulting from single mutations; however, the mutational origin of these human defects has not been directly demonstrated in any case. The most commonly demonstrated effect of mutation on enzymes is apparent loss. In a number of cases (71, 77, 79, 83, 97, 101, 107, 108, 112, 114, 121), the enzyme assay is sufficiently sensitive to exclude more than a fraction of 1 per cent of normal enzyme activity in the mutant. In several other cases (68, 117, 118, 122, 123), enzyme activity was detected in the mutant, but it was considerably less than the activity characteristic of the wild type. In order to justify the conclusion

TABLE I

Effects of Mutations on Enzymes*

Enzyme	Nutritional requirement or other effect	Reference
Escherichia coli		
Lipoic acid conjugase (L)	Lipothiamide pyrophosphate	(64)
Inosine phosphate dehydrogenase (L)	Guanine or xanthine	(65)
Adenylosuccinase (L)	Adenine	(66)
Dihydroorotate dehydrogenase (L)	Pyrimidine or orotate	(67)
Dihydroxyisovalerate dehydrase (L)	Isoleucine+valine	(68)
Isoleucine-valine-glutamate transaminase (L)	Isoleucine+valine	(69)
Threonine dehydrase (L)	Isoleucine or α-keto-butyrate	(70, 71)
Cystathionase (L)	Homocysteine or methionine	(72)
Diaminopimelic acid decarboxylase (L)	Lysine	(73)
Phenylpyruvate-forming enzyme (L)	Phenylalanine	(74)
Prephenic acid dehydrogenase (L)	Tyrosine	(75)
Dehydroshikimic acid reductase (L)	Mixture of aromatic compounds	(76)
Dehydroquinase (L, R)	Mixture of aromatic compounds	(77)
Tryptophan synthetase (L)	Tryptophan	(78)
Indole-glycerol-phosphate-forming enzyme (L)	Tryptophan	(78)
Indole-glycerol-phosphate-splitting enzyme (L)	Tryptophan	(78)
Histidinol dehydrogenase (L)	Histidine	(79)
Acetylornithinase (L)	Ornithine, citrulline, or arginine	(80, 81)
Pantothenic acid synthetase (L, A)	Pantothenic acid	(82)
Condensing enzyme (L)	α-Ketoglutarate	(83)
β-Galactosidase (L)	Inability to use lactose	(84)
Galactokinase (L)	Inability to use galactose	(85)
Galactose-1-phosphate uridyl transferase (L)	Ditto	(85)
Aerobacter aerogenes and *Salmonella typhimurium*		
Xanthosine phosphate aminase (L)	Guanine	(86)
Salmonella typhosa		
Xylose isomerase *and* xylulokinase (L)	Inability to use xylose	(87)
Pasteurella pestis		
Rhamnose isomerase *and* rhamnulokinase (G)	Ability to use rhamnose	(88)
Diplococcus pneumoniae		
Mannitol phosphate dehydrogenase (G)	Ability to use mannitol	(89)
Folic acid-forming enzyme (A)	Sulphonamide resistance	(13, 14)

TABLE I (*continued*)

Enzyme	Nutritional requirement or other effect	Reference
Achromobacter fischeri		
Luciferase (L)	Loss of luminescence	(90)
Bacillus subtilis		
Glutamic acid dehydrogenase (L)	An α-amino acid	(91)
Saccharomyces cerevisiae		
Diphosphothiamine phosphatase (I)	Thiamine	(92)
Galactokinase (L)	Inability to use galactose	(93)
Neurospora crassa		
Dihydroxyisovalerate dehydrase (R)	Isoleucine+valine	(68)
Transaminase (A)	Inhibition by threonine	(94)
Tyrosinase* (A)	None apparent	(95, 96)
Glutamic acid dehydrogenase (L, A)	An α-amino acid	(97 to 100)
Tryptophan synthetase (L, A)	Tryptophan	(101 to 106)
Argininosuccinase (L)	Arginine	(107)
Adenylosuccinase (L, A)	Adenine	(108, 109)
Pyrroline-5-carboxylate reductase (A)	Proline	(110, 111)
Imidazole glycerol phosphate dehydrase (L)	Histidine	(112)
Histidinol phosphate phosphatase (L)	Histidine	(113, 114)
Histidinol dehydrogenase (L)	Histidine	(114)
Cystathionase I (L)	Methionine, homocysteine or cystathionine	(115)
Cystathionase II (L)	Methionine or homocysteine	(115)
Nitrate reductase (L)	Inability to use nitrate	(116)
Oxaloacetate carboxylase (R)	Succinate or other Krebs cycle acid	(117)
Pyruvate carboxylase (R)	None normally apparent	(118)
Man		
Phenylalanine hydroxylase† (L)	Phenylketonuria	(119, 120)
Homogentisic acid oxidase† (L)	Alcaptonuria	(121)
Galactose phosphate uridyl transferase† (R)	Galactosemia	(122)
Glucuronide-synthesizing enzyme† (R)	Jaundice	(123)
Glucose-6-phosphatase†‡ (L, R)	Glycogen storage disease	(124)
Amylo-1,6-glucosidase†‡ (L)	Glycogen storage disease	(125)

* Key: (L) apparent loss; (G) apparent gain; (R) reduction in activity; (A) alteration in properties; (I) increase in activity.

† These variants discovered existing in populations; mutational origin not observed.

‡ These conditions thought to be caused by simple recessive mutations, though little genetic information is available (171).

that the effect of a mutation on growth is caused by an effect on the production of an enzyme, it is desirable to exclude two alternative possibilities; (a) that the mutation causes the production of an enzyme inhibitor; (b) that the reduced amount of enzyme found in the mutant is a consequence of the presence in the medium of the substance required for growth (126, 127). Alternative (a) has been excluded in numerous cases (e.g., 68, 75, 77, 79, 82, 85, 97, 107, 108, 121) by experiments showing that mutant extracts do not inhibit wild-type enzyme activity, while (b) is nearly always ruled out by the demonstration that the wild type or mutants blocked in other steps of the same biosynthesis are able to produce the enzyme on supplemented medium. Although loss or reduction of enzyme activity is the most commonly demonstrated case, the gain of an enzyme following mutation has been reported in at least two cases (88, 89), and the restoration of a lost enzyme by reverse mutation is frequently possible (103, 108, 128). Tavlitski (92) has reported an example, so far apparently unique, of a growth requirement which seems to result from an increase in an enzyme activity. A thiamine-requiring mutant of yeast was shown to produce abnormally high thiamine pyrophosphatase activity; the enzyme was shown to be inhibited by thiamine.

Of special interest are those cases where a qualitative alteration in an enzyme has been demonstrated. Maas & Davis (82) showed that an E. coli mutant requiring pantothenic acid for growth at temperatures above 30° produced an abnormally thermolabile pantothenate-synthesising enzyme. Experiments with mixtures of wild-type and mutant enzyme preparations, and studies of the kinetics of thermal inactivation, led to the conclusion that the abnormality resided in the enzyme molecule itself. Horowitz (95, 96) showed differences between wild strains of Neurospora in tyrosinase thermostability. Two sharply distinct types were found, apparently arising from two alleles at a single locus. The difference in thermostability was somewhat increased by partial purification of both types of enzyme, and, here again, the possibility that the difference was due to an impurity rather than to the enzyme itself was virtually ruled out. Heterocaryons carrying both types of nuclei produced tyrosinase which behaved as if it were a mixture of the two types of enzyme (96). A further example of a mutant apparently containing an excessively thermolabile enzyme (in this case adenylosuccinase) has been reported by Giles (109) and Giles and co-workers (108). Fincham (98) and Fincham & Pateman (99) have described a Neurospora mutant which produces glutamic dehydrogenase with an unusual type of temperature sensitivity. The mutant glutamic dehydrogenase had a very low activity in extracts prepared at low temperature, but it could be activated by mild heat treatment (2 min. at 35° sufficed); mutant extracts treated in this way had almost as much activity as wild-type extracts. The activation was completely reversible, and could also be partly achieved by incubation of the enzyme with substrates. Mixing experiments, and experiments on the kinetics of activation and loss of activity, indicated that the temperature sensitivity was a property of the enzyme itself rather than of an associated activator or

inhibitor, and this conclusion is supported by the observation that both wild-type and mutant enzymes can be considerably purified without alteration of their respective properties (129). Yura (111) has shown that a proline-requiring *Neurospora* mutant produces a pyrroline-5-carboxylate reductase characterised by an abnormally high energy of activation and also by reduced thermostability. Suskind (105, 106) has investigated a mutant of the *Neurospora td* series which requires tryptophan for growth at temperatures below 33°, and has shown that it produces a tryptophan synthetase which is abnormally sensitive to inhibition by a normal inorganic cell constituent which is almost certainly zinc. Mutations which alter the affinity of an enzyme for substrate, or for substrate analogues, seem rather rare, but Wagner & Ifland (94) have traced some of the peculiarities of a threonine-inhibited *Neurospora* mutant to its production of a transaminase with a reduced affinity for the α-keto analogue of valine. Hotchkiss & Evans have given a preliminary report on sulphonamide-resistant *Pneumococcus* mutants which produce forms of the folic acid-synthesising enzyme with reduced affinity for competitive inhibitors of the sulphonamide type (14). In all these cases where a mutation apparently causes a structural alteration in an enzyme, it is obviously desirable that the enzyme concerned should be purified and the nature of the difference determined in chemical and physical terms. This has not been achieved in any case described so far, though studies on the genetic control of a phosphatase in *E. coli,* of which a preliminary report has been given by Levinthal (130), seem to provide a good system for studies of this sort.

Effects on inducible enzymes and permeases.—Cohen & Monod (84) have demonstrated that in *E. coli* the uptake of sugars and amino acids from the growth medium depends on the presence in the cells of specific enzyme-like substances called permeases. They have shown that mutants unable to utilise lactose may be deficient either in the ability to form β-galactosidase or in the ability to form the specific β-galactoside permease, the formation of both normally depending on the presence in the cells of lactose or some other inducer. The two catalysts may be lost independently throught mutation at two distinct, though closely linked loci, while mutation at a third locus, closely linked to the other two, causes both the β-galactosidase and the permease to be produced in the absence of inducer (i.e., constitutively). The mode of action of this third locus is a problem. Vogel (126) has suggested that substances which cause specific induction or suppression of enzyme formation do so by facilitating or hindering the dissociation of the enzyme from its site of formation. If this is the case, a "constitutive" mutant might produce an enzyme altered in such a way as to dissociate spontaneously from its site of formation. The main difficulty with this hypothesis is that in the two cases where the enzyme of a constitutive mutant has been compared with the induced enzyme of the parent strain [β-galactosidase of *E. coli* (131), and penicillinase of *Bacillus cereus* (132)] the two have appeared identical in kinetic and immunological properties. An

alternative idea, which would explain how the same mutation could affect both the β-galactosidase and the permease, is that constitutive mutants produce an endogenous inducer.

Effects on proteins other than enzymes—Outstanding results have been obtained by Ingram and Hunt in the analysis of differences in amino acid sequence between different human haemoglobin types. The difference between normal haemoglobin (haemoglobin A) and sickle-cell haemoglobin (haemoglobin S) appears to be restricted to just one of the many peptides resulting from tryptic digestion (133, 134). The difference consists in the replacement of the sequence Thr-Pro-Glu-Glu-Lys in A by Thr-Pro-*Val*-Glu-Lys in S (135, 136). Analysis of another abnormal haemoglobin (haemoglobin C) has shown the abnormality to reside in the same position as in S, the sequence in this case being Thr-Pro-*Lys*-Glu-Lys (137). The three haemoglobin types probably correspond to three alleles at one locus. Numerous other varieties of human haemoglobin, thought to depend on further alleles at the same locus, are now known (138, 139, 140). Two kinds of haemoglobin, caused in each case by a difference at a single locus, have been reported both in sheep (141) and in cattle (142). In the case of the sheep haemoglobins, an investigation of amino acid composition has shown differences between the two types in respect of several amino acids (143), so the two molecules may differ at several points. Genetically controlled variation in serum proteins has been extensively studied both in man and in domestic animals. Smithies found by starch gel electrophoresis that human sera were of three types with respect to hemoglobin-binding proteins (haptoglobins) (144). Genetic data suggested that the three types corresponded to the two homozygotes and the heterozygote resulting from two alleles at one locus (145). A point of interest is that the presumed heterozygote forms at least one haptoglobin component not formed in either presumed homozygote; this may be a case of inter-allele complementation, which is discussed in the next section. Allison and his colleagues (146) have reported a fourth type of individual lacking all haptoglobins. Smithies (147) has reported three types of serum β-globulins from various human populations, and Harris *et al.* (148) have extended the number to five; multiple alleles at a single locus are probably responsible. β-Globulin polymorphism has also been described in sheep (149), while in cattle the presence or absence of a "slow-alpha" serum protein (150) and, among milk proteins, the occurrence of two β-lactoglobulins (151) and two α-lactalbumins (152), are all probably controlled by single loci.

Biochemical effects of cytoplasmic mutations.—The only cytoplasmically inherited differences which have been studied biochemically are those causing respiratory deficiencies in *Neurospora crassa* and yeast. The work of Slonimski, Ephrussi and co-workers, and Yotsuyanagi (153 to 156) on cytoplasmically inherited respiratory deficiency in yeast is probably too well known to need reviewing here. In *Neurospora* several types of mutant with respiratory deficiencies have been described; various kinds of cytoplasmic

mutation as well as chromosomal mutation at, at least, two different loci are responsible in different cases (157, 158). The various mutants have characteristic abnormalities in their cytochrome components, and this has led to new information on respiratory mechanisms in *Neurospora* (158, 159, 160). The experiment of combining two different defective cytoplasms by vegetative hyphal fusion has given different results in the two cases in which it has been tried. In one case a complementary interaction with the formation of an almost normal mycelium has been reported (161), while in the other, in which another combination of cytoplasmic mutants was used, no interaction was evident (162). There thus appear to be several possible hereditary cytoplasmic states in *Neurospora*, each with is own characteristic abnormality of the cytochrome system, but the nature of the genetic factors involved and the relation between them are not at all understood. The phenomenon of "long-term adaption" in yeast, described by Campbell & Spiegelman (163), seems likely to be an example of what might be termed "spurious cytoplasmic inheritance." In the presence of galactose during growth, ability to ferment this sugar has the appearance of a mutation, while the loss of this ability during growth in its absence proceeds as if caused by the diluting out of a cytoplasmic particle. Campbell & Spiegelman postulate an enzyme-forming system consisting of a template-enzyme-inducer (galactose) complex, so that the enzyme would be autocatalytic during adaptation, and the active enzyme-forming complexes would be diluted out during growth in the absence of galactose. However, it seems possible to make an alternative interpretation in terms of the autocatalytic properties of an inducible galactose permease system. As Novick & Weiner (164) have demonstrated, when cells lacking a permease are exposed to a substrate whose uptake depends on the permease and which acts as an inducer of it, the rare uptake of a substrate molecule will simulate a mutation followed, in turn, by a rapid and apparently autocatalytic build-up of permease and hence of ability to utilise the substrate. On this interpretation the particles diluted out during growth in absence of substrate could be permease molecules.

SPATIAL ORGANIZATION OF GENETIC MATERIAL IN RELATION TO FUNCTION

In organisms in which the location of chromosomal mutations can be established, there is a strong suggestion that all mutations affecting the production of the same protein tend to be located within the same chromosome region. In *Neurospora* this seems to be true in the cases of mutations affecting adenylosuccinase (108, 109), glutamic dehydrogenase (99, 129), and argininosuccinase (107, 165), and more limited genetic data on mutants lacking enzymes for histidine synthesis (112, 113, 114) point in the same direction. Studies on mutants lacking tryptophan synthetase (101 to 105) have revealed a more complicated situation. While mutations at only one locus were capable of causing qualitative alteration in, or loss of the enzyme, it was shown that "suppressor" mutations at other loci could partially restore

enzyme activity in certain of the enzyme-deficient mutants (103). There was some correlation between the "suppressibility" of mutants and their ability to produce a protein ("cross-reacting material") immunologically related to tryptophan synthetase but lacking its activity (166). A possible explanation of the action of suppressor mutations is that they are not concerned with the formation of the enzyme but rather bring about alterations in intracellular conditions so as to enable abnormal forms of the enzyme to show activity. Suskind (105, 106) has supporting evidence, in the mutant mentioned above, which produces an abnormally zinc-sensitive tryptophan synthetase; a suppressor mutation promotes enzyme activity (probably by reducing intracellular zinc concentration) without altering the properties of the enzyme itself. Drastic reductions in tyrosinase activity in the mould *Glomerella cingulata* (167, 168) and in lactase activity in *Neurospora* (169, 170) have been found as a result of mutation at any one of several different loci in each case. In both cases the formation of the enzyme was critically dependent on the composition of the growth medium, and susceptibility to various kinds of genetic alteration is perhaps to be expected in these circumstances. Three distinct loci in *Neurospora* can mutate so as to cause failure of nitrate reductase formation. According to Silver & McElroy (116), one mutant contained an inhibitor of nitrate reductase, so the effect on the enzyme in this case is probably indirect. In *Drosophila* Glassman *et al.* (172) have found that the nonallelic mutants *rosy* and *maroonlike* are both deficient in xanthine dehydrogenase. While the generalisation that the formation of an enzyme can be affected by mutation at one locus only is evidently not valid, it may still be true that the *structure* of a given protein depends on but a single locus. Too few cases of qualitative genetically determined alterations in proteins are known as yet for adequate testing of the latter hypothesis, which is, however, susceptible to experimental test.

There remains the question whether loci corresponding to different proteins are always spatially distinct, or whether one can find overlapping loci or loci with more than one function. In considering this question, one should bear in mind the likelihood of secondary effects of mutations on enzymic activities. For example, Gross (174) has reported that a *Neurospora* mutant deficient in dehydroshikimic acid reductase (presumably the primary effect of the mutation) accumulated dehydroshikimic and protocatechuic acids with the consequent induced formation of dehydroshikimic acid dehydrase and protocatechuic acid oxidase; these enzymes are not detectable in the wild type grown under the same conditions. On the whole, however, each mutation seems to have a major effect on only a single enzyme. Numerous cases are on record (e.g., 65 to 69, 83, 85, 93, 97, 112, 121) where only one enzyme in a metabolic sequence has been lost after mutation and the other enzymes of the series have remained untouched. This specificity of effect can provide the biochemist with a valuable tool for the resolution of enzymes with similar or related functions (71, 110). Where mutants have multiple growth requirements this may result from a single block in the synthesis of a

common precursor (e.g., 173) or from the loss of a single enzyme involved in more than one biosynthesis (68, 69). In several cases two steps in the same pathway have been shown to depend on a single genetic locus. Both in *Neurospora* (108) and in *E. coli* (66) it has been shown that a single mutation eliminates two enzyme activities in the biosynthesis of adenine, one the splitting of succinyl-4-amino-imidazole-carboxamide ribotide and the other the splitting of adenylosuccinic acid. Since the two reactions are quite similar, it seems reasonable to suppose that the same enzyme catalyses both. Yanofsky has shown that in *E. coli* one type of tryptophan-requiring mutant lacks *both* tryptophan synthetase and the enzyme catalysing the formation of indole from indole glycerol phosphate (78). A second type of mutant, which lacked only the tryptophan synthetase activity, produced a protein which was immunologically indistinguishable from tryptophan synthetase (175) and which possessed the indole-forming activity (176). It seems here either that a single genetic locus is involved in the formation of two related proteins or (perhaps more likely) that a single protein catalyses the two sequential reactions and that mutations at a single locus can eliminate one or both of its activities in different cases. In *Pasteurella pestis* the capacity to form two enzymes necessary for rhamnose utilisation, rhamnose isomerase and rhamnulokinase, has been reported by Englesberg (88) to be gained in a single mutational step, while Kline & Baron (87) have described an apparently analogous situation with regard to xylose isomerase and xylulokinase in *Salmonella*. Since in these two instances the enzymes concerned are induced by substrate it is possible to suggest that the primary effects of the mutations are on substrate penetration, but here again the possibility that two sequential reactions are catalysed by a single protein deserves consideration.

Various workers, among them Hartman (2), have shown that in *Salmonella* chromosomal loci concerned with the formation of different enzymes in the same pathway are often closely linked. In the best investigated case, that of histidine biosynthesis, six closely-linked loci are arranged in an order on the chromosome corresponding to the sequence of action of the enzymes which they control (177). So far as it is known, these loci are not separated from each other by other loci of unrelated function. A similar ordered sequence of loci concerned in tryptophan synthesis occurs in *Salmonella* (178) and also, probably in *E. coli* (78). Other cases in *E. coli* of close linkage of loci concerned in sequential metabolic steps are those concerning galactokinase and galactose-1-phosphate uridyl transferase (85, 179, 180), and galactoside permease and β-galactosidase (84). The tendency for loci of related function to be linked is not nearly so apparent in *Neurospora*, where loci analogous to those controlling histidine synthesis in *Salmonella* occur scattered over different chromosomes (112, 113, 114, 181). There are, however, indications of nonrandom arrangement of loci in *Neurospora;* one may cite the relatively close linkage of pairs of loci concerned, respectively, with synthesis of carotenoid pigments (181), isoleucine and valine (181),

cysteine (182), and adenine (183). An important question raised by these results is whether it is always possible to make a sharp distinction between "single enzymes," some of which may catalyse more than one step in a metabolic pathway (see above), and organized arrays of enzymes such as are suggested by the *Salmonella* work. If such a distinction is not always possible, then the concept of a genetic locus as a functional unit becomes correspondingly less clear cut.

A surprising type of interaction between alleles in the formation of single enzymes has been reported for three *Neurospora* loci, concerned respectively with glutamic dehydrogenase (99, 129, 184), adenylosuccinase (108, 109) and tryptophan synthetase (185), and for two *Salmonella* loci, concerned respectively with L-histidinol dehydrogenase and imidazole glycerol dehydrase (177). In all these cases certain pairs of alleles, individually incapable of promoting formation of the enzyme concerned, are capable of doing so when brought together in the same cell by heterocaryon formation (in *Neurospora*) or by phage-mediated abortive transduction (in *Salmonella*). This type of interallele complementation has not so far been found to give more than about a quarter of the typical wild-type enzyme level in any case. In all the examples studied it has been found possible to represent the relationships of the various alleles at a locus by a linear "complementation map," noncomplementary pairs being represented by overlapping and complementary pairs by nonoverlapping segments of the map. Giles (109) has preliminary evidence that such a map may be correlated with the linear fine structure of the locus as determined by orthodox crossing-over analysis. Whether interallele complementation should be interpreted as indicating that the enzymes concerned can be formed in several steps, or by the assembly of preformed polypeptides, or whether the complementary interaction occurs rather at the level of the enzyme-forming system (perhaps between pieces of template RNA) is not at present clear. The data do, however, argue strongly against a direct synthesis of enzyme by a chromosomal locus. They also demonstrate that the type of chromosomal unit which we have been calling "locus" is not necessarily a cistron as defined by Benzer (3).

Perhaps the most intriguing possibility at the present time is that of correlating the arrangement of different mutations within a locus, as determined by genetic mapping (3, 186), with the effects of the mutations on the amino acid sequence in the corresponding protein (133 to 137). The recent work of Levinthal (130) on the genetic control of phosphatase in *E. coli* shows promise of advance in this direction. The prospect of being able to determine the fine structure of the genetic material in the chemical rather than in the formal genetical sense seems much more remote. Studies on the transforming principles of *Pneumococcus* (e.g., 13) seem to offer the best hope, but substantial progress will depend on the development of new methods for fractionating and characterising nucleic acids.

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NUCLEIC ACIDS, PURINES, PYRIMIDINES (NUCLEOTIDE SYNTHESIS)[1,2]

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During the years 1956 and 1958 the chapters on nucleosides, nucleotides, and nucleic acids were developed with emphasis on the enzymological studies (1, 2). Although the authors have this year been requested to develop particularly the literature relating to nucleotide synthesis, detailed information on the enzymatic synthesis of polynucleotides has appeared during this period and has been included. The factors involved in nucleotide and nucleic acid synthesis *in vivo* have also been discussed. With certain exceptions, extensive discussion has been omitted of topics related to the particition of nucleotides in other metabolic processes to be reviewed in this volume, such as protein synthesis and carbohydrate metabolism. Also excluded is the extensive literature on the chemical synthesis of compounds, some of which are potential inhibitors of nucleic acid metabolism.

The Biosynthesis of Pyrimidines

The contributions to the development of the field of pyrimidine biosynthesis have been concerned chiefly with (*a*) the synthesis of carbamyl phosphate, (*b*) the amination of uridylic to cytidylic acid, (*c*) the conversion of deoxyuridylic and deoxycytidylic acids to their corresponding methyl or hydroxymethyl derivatives, and (*d*) the possibility that more than one pathway for pyrimidine biosynthesis exists.

Hall *et al.* (3) have isolated and characterized as acetylglutamic acid the naturally occurring cofactor of carbamyl phosphate biosynthesis. This compound has been isolated from both liver and yeast. Although acetylglutamate (or other N-substituted derivatives of glutamic acid) has been

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[1] The survey of the literature pertaining to this review was completed in November 1958.

[2] The following abbreviations are used in this chapter: RNA for ribonucleic acid; DNA for deoxyribonucleic acid; AMP, ADP, ATP, GMP, GDP, GTP, IMP, IDP, ITP, CMP, CDP, CTP for the mono-, di- and triphosphates of adenosine, guanosine, inosine and cytidine; UDP for uridine diphosphate; TPN, TPNH, DPN, DPNH for the oxidized and reduced forms of triphosphopyridine nucleotide and diphosphopyridine nucleotide; THFA for tetrahydrofolic acid. N^5,N^{10}-anhydroformyltetrahydrofolic acid, an imidazolinium compound, has also been referred to as 5,10-methenyltetrahydrofolic acid, anhydroleucovorin, or isoleucovorin.

established as a required cofactor for carbamyl phosphate synthesis in mammalian liver, no such requirement has been demonstrated for the corresponding reaction in microorganisms. The role of acetylglutamate in the metabolism of cells other than liver is still unknown.

Ravel *et al.* (4) have explored the participation of biotin in the carbamylation reaction. The carbamylations of ornithine in citrulline synthesis and of aspartic acid in pyrimidine synthesis are severely reduced in cell-free extracts of biotin-deficient *Lactobacillus arabinosis,* and are not restored by the addition of biotin or heat-inactivated extracts of normal cells. However, normal activity may be found in extracts of deficient cells grown for only a short time in a biotin-supplemented growth medium. Purified preparations of the ornithine-citrulline enzyme from *Streptococcus lactis* contain only small amounts of biotin. Ravel *et al.* conclude therefore that biotin is involved in the synthesis of the carbamylation enzyme but is not directly utilized as a cofactor in the synthesis or utilization of carbamyl phosphate.

Mokrasch & Grisolia (5) and Mokrasch *et al.* (6) have examined the compounds carbamyl β-alanine ribotide, dihydrouridine-5′-phosphate and uridine-5′-phosphate as precursors of RNA in chicken liver preparations. All compounds were superior to orotic acid or to the corresponding compounds lacking the phosphate or phosphoribose moieties. The conclusion drawn from these experiments was that pyrimidine nucleotides may be synthesized from elementary intermediates such as carbamyl β-alanine ribotide by a route which excludes the orotic acid pathway. Final recognition of an alternate scheme to the already established pathway in animal and microbial systems involving carbamyl aspartic, dihydroorotic, orotic, and orotidylic acids as intermediates will require further clarification of the enzymatic processes and an understanding of the metabolic origin of carbamyl β-alanine ribotide. In this connection Boyd & Fairley (7) have now shown that propionic acid as well as α-aminobutyric acid (8) is utilized as a pyrimidine precursor by a pyrimidineless mutant of *Neurospora crassa.* They suggest that these compounds are metabolized to pyrimidines via an activated derivative of β-alanine.

Weed (9) has reported evidence for the synthesis of a new compound, presumably a nucleotide, which is formed from either orotic acid or carbamyl aspartic acid by rat liver slices and which is incorporated into the acid-insoluble fraction. The identity of the compound has not been established.

In contrast to the bacterial system described by Lieberman (10), the enzyme from mammalian tissue utilizes glutamine instead of ammonia as the nitrogen donor of the amino group in the conversion of uridylic to cytidylic acid. This difference was anticipated by the findings of Eidinoff *et al.* (11), whose experiments with the glutamine analogues, azaserine and 6-diazo-5-oxonorleucine, suggested that glutamine was a participant in the reaction. Salzman *et al.* (12) have shown that the amino nitrogen of cy-

tidine of RNA and DNA of HeLa cells can be supplied by the amide nitrogen of glutamine but not by ammonia. Kammen & Hurlbert (13, 14) have now isolated a soluble enzyme system from a rat tumor (the Novikoff hepatoma) which requires uridylic acid, ATP, glutamine, and a guanosine nucleotide. It is not certain what the exact functions of the individual nucleotide compounds are. It is possible that uridine triphosphate is the actual compound aminated, but the enzyme system is not sufficiently purified to determine this conclusively.

The problem of the synthesis of the methyl group of thymine continues to receive considerable attention. In continuation of an earlier report from their laboratory (15), Greenberg & Humphreys (16) have shown that a direct relationship exists between the concentration of tetrahydrofolic acid and the formation of thymidylic acid from deoxyuridylic acid and formaldehyde in an enzyme system from thymus. Reduced diphosphopyridine nucleotide stimulates methyl group synthesis by tetrahydrofolic acid when the concentration of the latter is below that required for a maximal rate of synthesis. Attempts to determine the pathway of thymidylic acid formation in normal and neoplastic cell suspensions have been reported also by Kit et al. (17).

In continuation of previously reported work on this reaction in microorganisms (18), Birnie & Crosbie (19) have demonstrated the formation of thymidylic acid in a cell-free extract of a serineless mutant of Escherichia coli from deoxyuridine, serine, ATP, tetrahydrofolic acid, and reduced TPN. Dinning et al. (20) have carried out experiments with C^{14}-formate in Lactobacillus leichmannii which indicate that B_{12} is involved in the conversion of formate to thymine methyl groups. Their data are interpreted to exclude as intermediates serine, methionine, or hydroxymethyl compounds with which these amino acids may be in equilibrium. Also, in contrast to the report of Wagle, Mehta & Johnson (21), no evidence for a role of B_{12} in protein biosynthesis could be demonstrated. On the other hand, Helleiner, Kisliuk & Woods (22) have obtained a cell-free system from a mutant of E. coli in which B_{12} is required for the enzymatic synthesis of methionine from serine. Tetrahydrofolic acid is a necessary factor of this reaction.

Further data have been reported on the interesting problem of the synthesis of pyrimidine nucleotides in cells infected with bacteriophage. Amos & Magasanik (23) have shown that cells of E. coli infected with T7 bacteriophage will utilize uridine-C^{14} in the synthesis of thymidylic acid of the deoxyribonucleic acid and that the uridine ribose is the source of thymidylic acid deoxyribose. Flaks & Cohen (24) have reported further investigations on the enzyme, deoxycytidylate hydroxymethylase, which is found in extracts of E. coli B infected with any bacteriophage containing hydroxymethylcytosine, i.e., the T-even phages. This enzyme is responsible for the formation of 5-hydroxymethyl deoxycytidylic acid from formaldehyde and deoxycytidylic acid in the presence of tetrahydrofolic acid. The enzyme activity was absent from extracts prepared from uninfected cells

disrupted in a number of ways and from extracts of cells infected with T1 phage. The enzyme activity was also absent from intact or osmotically disrupted T6 virus itself. Essentially normal production of enzyme was obtained when cells were infected with heavily irradiated T6 under conditions where multiplications or appreciable multiplicity reactivation of the bacteriophage was not observed.

BIOSYNTHESIS OF PURINE NUCLEOTIDES DE NOVO

Enzymatic steps in purine nucleotide synthesis.—Because of the complexity of the enzymatic process of inosinic acid biosynthesis from its elementary precursors these reactions are summarized in Figure 1. Most of the individual steps have been treated in previous Reviews, but for the sake of completeness some of the important references pertaining to each of these reactions will be given here: ribose-5-phosphate to phosphoribosylpyrophosphate (25, 26), phosphoribosylpyrophosphate to phosphoribosylamine (27, 28), phosphoribosylamine to glycinamide ribotide (27, 29), glycinamide ribotide to formylglycinamide ribotide (30, 31), formylglycinamide ribotide to formylglycinamidine ribotide (32), formylglycinamidine
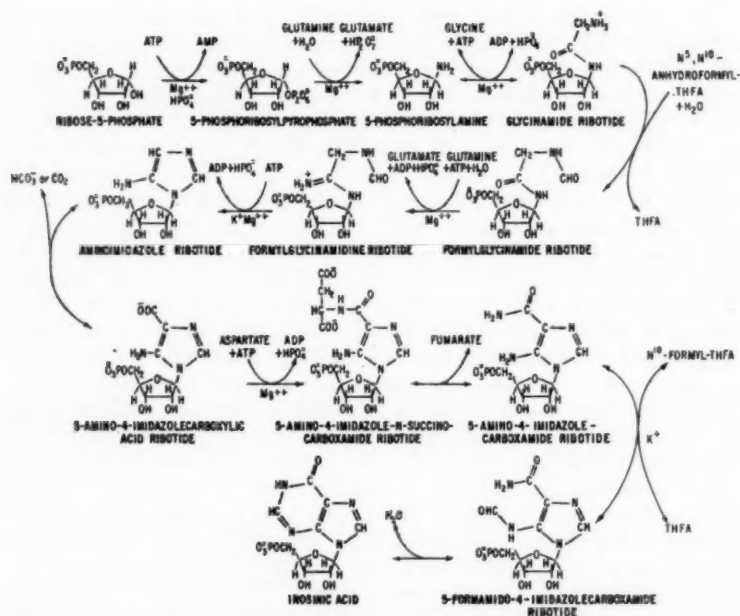
FIG. 1. Biosynthesis of Inosinic Acid.

ribotide to aminoimidazole ribotide (32), aminoimidazole ribotide to amino-imidazolecarboxylic acid ribotide (33), aminoimidazolecarboxylic acid ribotide to aminoimidazole-N-succinocarboxamide ribotide (33), aminoimidazole-N-succinocarboxamide ribotide to aminoimidazolecarboxamide ribotide (34), aminoimidazolecarboxamide ribotide to inosinic acid (35, 36).

Within the past year certain of these reactions have been studied in more detail. The formation of phosphoribosylamine from phosphoribosyl-pyrophosphate and glutamine previously reported by Goldthwait (27) has been investigated with an enzyme system purified approximately one-hundredfold from pigeon liver (28). During the reaction, which is apparently irreversible, glutamic acid and pyrophosphate are formed at essentially equal rates. There is no evidence that an intermediate such as a glutaminyl ribotide is involved. The product of this reaction, phosphoribosylamine, has as yet not been isolated from an enzymatic system. The further conversion of this intermediate, formed by chemical synthesis, has been studied with partially purified avian liver enzymes. The reversible reaction between phosphoribosylamine, glycine, and ATP appears in all respects to be analogous to the synthesis of glutamine from glutamic acid, ammonia, and ATP (29). Since no synthetic or exchange reactions can be shown in either of these systems in the absence of any one of the three substrates, and because arsenolysis of the amide products requires ADP, a mechanism was proposed in which the concerted participation of all of three substrates in either reaction is required in the bond-forming steps.

(a) *Guanylic acid synthesis.*—Magasanik and co-workers (37, 38), Lagerkvist (39, 40), and Abrams & Bentley (41) have reported on the conversion of inosinic acid to guanylic acid in bacterial, avian, and mammalian systems, respectively. In all of these systems the initial enzymatic step is the oxidation of inosine-5'-phosphate in the presence of the DPN-linked enzyme, inosine-5'-phosphate dehydrogenase. The oxygen atom introduced in this reaction is presumably derived from water.

With an enzyme preparation purified three-hundredfold from *Aerobacter aerogenes,* Moyed & Magasanik (38) had previously shown that ammonia rather than ammonium ions reacted with xanthosine-5'-phosphate to yield guanosine-5'-phosphate and that ATP was split to AMP and pyrophosphate in the process. The corresponding aminating enzyme has been purified ninetyfold from pigeon liver (40) and has been shown to catalyze the reaction whose stoichiometry is shown in Reaction 1.

xanthosine-5'-phosphate + ATP + glutamine + H$_2$O ⟶ guanosine-5'

$$\text{phosphate} + \text{glutamate} + \text{AMP} + \text{pyrophosphate} \qquad (1)$$
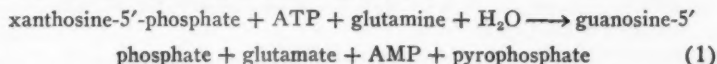
Neither glutamic acid nor asparagine could substitute for glutamine, but ammonium ions were utilized to a small extent as the aminating agent. Labeled glutamate and pyrophosphate were not incorporated under any conditions into glutamine and ATP, respectively. This experiment demonstrated the virtual irreversibility of this reaction. Studies with O^{18} showed

that oxygen from the 2-position of xanthosine-5′-phosphate was transferred to the phosphate group of the AMP formed in this reaction. This observation suggested the possibility that an adenyl-xanthosine-5′-phosphate was involved as an intermediate in guanylic acid biosynthesis. The enzyme does catalyze an exchange of pyrophosphate with ATP, but this exchange is independent of added xanthosine-5′-phosphate. Upon further purification of the enzyme, the activity responsible for the pyrophosphate exchange was partially separated from the aminating activity. There is, therefore, some doubt whether the pyrophosphate exchange reaction is directly concerned with the amination of xanthylic acid and whether adenyl-xanthosine-5′-phosphate is involved as an intermediate of the reaction.

Nearly identical results have been obtained by Abrams & Bentley (41), who employed a purified enzyme from calf thymus in their studies. The apparent K_m values for glutamine and ammonium ions are 5×10^{-4} M and 3.9×10^{-2} M, respectively, at pH 7.65. The latter value is in approximate agreement with that found for ammonium ions in the bacterial system. Similar results to those of Lagerkvist were obtained in an isotopic oxygen transfer experiment. It could be shown that the pyrophosphate exchange reaction catalyzed by the crude enzyme could be almost completely removed upon subsequent purification. The authors conclude that adenyl-xanthosine-5′-phosphate is therefore an unlikely intermediate, but that a concerted participation of ATP and the amino donor, glutamine, are required to cleave the C—O bond of xanthosine-5′-phosphate and to form simultaneously the C—N bond of guanosine-5′-phosphate.

(b) Conversion of guanylic acid to adenylic acid in bacteria.—Mager & Magasanik (42) have reported that the conversion of guanine compounds to nucleic acid adenine in *E. coli* does not involve a reversal of the pathway of guanylic acid formation from inosinic acid but that the reductive deamination of guanosine-5′-phosphate to inosine-5′-phosphate in the presence of TPNH is a key reaction in this conversion (Reaction 2).

$$\text{guanosine-5′-phosphate} + \text{TPNH} + \text{H}^+ \longrightarrow \text{inosine-5′-phosphate}$$
$$+ \text{TPN}^+ + \text{NH}_3 \qquad (2)$$
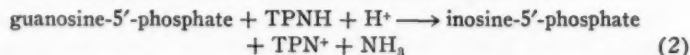
The reaction is irreversible in the presence of an appropriate TPNH regenerating system. Presumably the inosine-5′-phosphate is then converted to adenosine-5′-phosphate by the reactions described previously by Lieberman (43), and by Carter & Cohen (44) (Reactions 3 and 4).

$$\text{inosine-5′-phosphate} + \text{GTP} + \text{aspartic acid} \rightleftharpoons \text{adenylosuccinic}$$
$$\text{acid} + \text{GDP} + \text{HPO}_4^= \qquad (3)$$
$$\text{adenylosuccinic acid} \rightleftharpoons \text{adenosine-5′-phosphate} + \text{fumaric acid} \qquad (4)$$
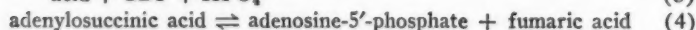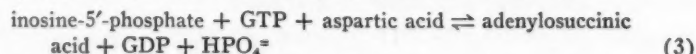
(c) Mechanism of adenylosuccinic acid synthesis.—The mechanism of the enzymatic formation of adenylosuccinic acid (Reaction 3) has been studied by Fromm (45), who compared rates of exchange of aspartic acid with adenylosuccinic acid and of phosphate with GTP at equilibrium. The

finding that the aspartic acid exchange was more rapid than that of phosphate was taken as support for the hypothesis, based on oxygen transfer studies, that 6-phosphorylinosine-5'-phosphate is an intermediate in the reaction (43). If this assumption is correct, it would be expected that the exchange of aspartic acid with adenylosuccinic acid would require only phosphate and not GDP. Since this exchange is enhanced over thirtyfold in the presence of GDP, a more likely mechanism for this reaction might involve the simultaneous participation of both the nucleoside triphosphate and the amino group of aspartic acid at the 6-position of inosinic acid rather than a pathway including a phosphorylinosinic acid intermediate.

Purines and one-carbon metabolism.—Recent work with isolated enzyme systems has shed considerable light on the processes of "one-carbon" metabolism. These interconversions in "one-carbon" metabolism are mediated by a few key derivatives of tetrahydrofolic acid. It has been known that carbohydrates are probably the eventual source of the major portion of this "one-carbon pool" in many systems by virtue of their conversion to phosphohydroxpyruvic acid, phosphoserine, and then serine. The reversible formation of glycine from serine involves the transfer of the β-hydroxymethyl group of serine to tetrahydrofolic acid. The structure of this hydroxymethyl-containing intermediate is not known with certainty but is believed by Huennekens *et al.* (46) to be N^5, N^{10}-methylenetetrahydrofolic acid. Formaldehyde can be reacted enzymatically or nonenzymatically with tetrahydrofolic acid to yield the same derivative and many thereby enter the pool. Enzymatic reduction of this active "hydroxymethyl" compound is presumably involved in the synthesis of the methyl groups of methionine and thymidylic acid.

Osborn & Huennekens (47) have shown that oxidation of the hydroxymethyltetrahydrofolic acid derivative to one at the formate level of oxidation requires TPN. After removing interfering enzymes, they were able to show that the initial product of this oxidation was the N^5, N^{10}-anhydroformyl derivative of tetrahydrofolic acid. This compound may be formed from formiminoglutamic acid produced in histidine catabolism or from formiminoglycine produced in the fermentation of purines (48).

Formate may be converted to an activated form by reaction with tetrahydrofolic acid and ATP in the the presence of the enzyme, tetrahydrofolic formylase. The immediate products of this reaction are N^{10}-formyltetrahydrofolic acid, ADP, and inorganic phosphate (35). Rabinowitz & Pricer (49) have succeeded in crystallizing this enzyme from *Clostridium cylindrosporum* and have described convenient assays for the determination of formate, ATP, or the enzyme itself by use of this reaction (50). The reaction could be shown to be reversible if the ATP formed were removed by utilization in glucose phosphorylation.

The mechanism of this activation process has been studied both by Greenberg & Jaenicke (51, 52) and by Whiteley *et al.* (46, 53). When studied with highly purified enzyme preparations from liver or bacteria,

the reaction seems to proceed via a free phosphorylated derivative of tetrahydrofolic acid. Upon the enzymatic incubation of ATP and tetrahydrofolic acid, a phosphorylated derivative of tetrahydrofolic acid was formed which could be isolated by paper chromatography. If this material were reincubated with formate in the presence of the same enzyme, N^{10}-formyltetrahydrofolic acid was produced. It has been suggested that the intermediate is N^{10}-phosphoryltetrahydrofolic acid (46), although definite proof of this structure is still lacking. Final acceptance of this mechanism should await the definite identification of this intermediate since it is somewhat difficult to visualize the formation of an N^{10}-formyl product from an N^{10}-phosphoryl intermediate in terms of our present understanding of phosphate-activated processes.

The interconversion of these two forms of "active formate," i.e., N^5, N^{10}-anhydroformyl- and N^{10}-formyltetrahydrofolic acid, not only is catalyzed by the enzyme, cyclohydrolase (48), but also proceeds at a slower rate nonenzymatically. Studies by Warren, Flaks & Buchanan (54), using purified preparations of glycinamide ribotide transformylase and 5-amino-4-imidazolecarboxamide ribotide transformylase, showed that either of these two folic acid derivatives would serve as formyl donors in both enzyme systems. Because of the interconversion of the formyl cofactors, it was not possible to decide which derivative was the immediate reactant in the formate incorporation reactions of purine biosynthesis. It has been possible recently to obtain enzyme preparations free from cyclohydrolase which show definite preferences with respect to the formyltetrahydrofolic acid derivative (55). Glycinamide ribotide transformylase specifically requires N^5, N^{10}-anhydroformyltetrahydrofolic acid as a formyl donor while aminoimidazolecarboxamide ribotide transformylase requires N^{10}-formyltetrahydrofolic acid (see Figure 1). The two transformylation reactions of purine biosynthesis may be linked in the direction shown in Reaction 5. However, the enzyme cyclohydrolase must be present to convert N^{10}-formyltetrahydrofolic acid to N^5, N^{10}-anhydroformyltetrahydrofolic acid.

$$\text{THFA}$$

inosinic acid + glycinamide ribotide + $H_2O \longrightarrow$ aminoimidazolecarboxamide ribotide + formylglycinamide ribotide   (5)
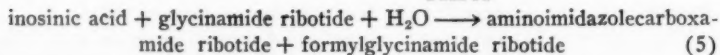
In studies on the metabolism of $2\text{-}C^{14}$-histidine in bacteria, Revel & Magasanik (56) found that species of Pseudomonas which degrade histidine to formate incorporated the labeled carbon of the precursor into purines while species of Aerobacter and Salmonella which produce formamide from histidine did not. With labeled histidine and formate as precursors, the purines were labeled only in position-8 while with $2\text{-}C^{14}$-glycine the purines contained C^{14} equally in positions 2 and 8. The conversion of $2\text{-}C^{14}$-histidine to purines did not proceed via free formate. "One-carbon" metabolism, at least as far as purines are concerned, seems to differ in these bacteria from that encountered in animal systems.

Possibility of alternate routes of purine synthesis.—The pathway of purine synthesis outlined in Figure 1 has been studied primarily with avian liver enzyme systems in which this process is predominantly a mechanism of nitrogen excretion. It is conceivable that alternate routes or variations from that shown in Figure 1 might be possible in organisms in which purine synthesis is mainly directed toward nucleic acid synthesis. Studies with microorganisms have suggested that alternate pathways do not exist but that the pathway of Figure 1 is general. Various workers have shown that purine-requiring mutants of *E. coli* and *A. aerogenes* accumulate derivatives of aminoimidazole (57), 5-amino-4-imidazole-N-succinocarboxamide (58), 5-aminoimidazolecarboxamide (59), and xanthine (37). An adenine-requiring mutant which is unable to cleave adenylosuccinic acid to adenylic acid accumulates derivatives of 5-amino-4-imidazole-N-succinocarboxamide ribotide (58) since the single enzyme which splits both of these succino compounds is missing. Lones *et al.* (60) have reported that yeast grown under conditions of biotin deficiency excrete aminoimidazole riboside and hypoxanthine. The inhibition of purine synthesis and the accumulation of formylglycinamide ribotide in *E. coli* (61) is caused by azaserine. Crude extracts of *E. coli* are also able to convert 5-phosphoribosylpyrophosphate, glutamine, glycine, and ATP to glycinamide ribotide. Cell-free extracts of *N. crassa* have been shown capable of catalyzing all of the reactions of Figure 1 (62). It seems probable that the other intermediates of purine biosynthesis and the reactions of their interconversion are essentially the same as those of pigeon liver. Magasanik *et al.* (37, 38) have provided evidence from studies with mutants of *A. aerogenes* that there are no alternate pathways for guanylic acid biosynthesis in this species. The only known difference between the avian and bacterial routes of purine formation is the difference in the aminating agent required for guanylic acid synthesis, a difference which seems to be repeated in the amination reactions in the formation of cytidine compounds (10, 14). Glutamine has been shown to be the nitrogen donor in the conversion of formylglycinamide ribotide to formylglycinamidine ribotide with the enzyme system obtained from *E. coli*, *Salmonella typhimurium* and *Saccharomyces cerevisiae* (62).

Mechanisms for the control of purine synthesis.—Magasanik (63) has reviewed the problem of control mechanisms in the metabolism of microorganisms. The suppression exerted upon the synthesis of a metabolite by the metabolite itself or a related material can be achieved either by interfering with enzyme synthesis or enzyme action. In the case of purine synthesis, Magasanik, Moyed & Gehring (37) have demonstrated the striking effect of guanine on the formation of inosine-5'-phosphate dehydrogenase in bacterial mutants lacking xanthosine-5'-phosphate aminase. Several possible control mechanisms exist at the metabolic level. Moyed & Magasanik (38) have proposed that the reciprocal requirement for ATP in guanosine-5'-phosphate synthesis and of GTP in adenosine-5'-phosphate formation may control the rate of synthesis and concentration of these nucleic acid

precursors. Isotopic and nutritional studies have shown that adenine and guanine compounds are readily interconvertible in bacteria. Mager & Magasanik (42) have recently shown that the conversion of GMP to adenine compounds proceeds via reductive deamination of GMP to IMP (see Reaction 2). The adenine derivative, ATP, markedly inhibits this enzymatic process. Thereby the synthesis of adenosine compounds from guanine nucleotides may be regulated by the concentration of adenosine compounds in the cell. In studies on bacterial mutants lacking the enzyme which converts 5-amino-4-imidazole-N-succinocarboxamide ribotide to aminoimidazole carboxamide ribotide Gots & Gollub (58) have found that the accumulation of the succino compound is inhibited by exogenous adenine. Similarly, the accumulation of aminoimidazolecarboxamide riboside by a mutant of *E. coli* is suppressed by a large number of purine compounds (59).

Feedback control of purine biosynthesis by product inhibition has recently been reported in studies with pigeon liver extracts. Wyngaarden, Silberman & Sadler (64) found that a number of purines and purine derivatives, including adenine, xanthine, inosine, IMP, IDP, ITP, and aminoimidazole carboxamide, its riboside and ribotide, inhibited *de novo* purine formation, but AMP and GMP did not. If phosphoribosylpyrophosphate were supplied, xanthine and IMP no longer inhibited, and none of the compounds affected the conversion of glycinamide ribotide to IMP. The effect of the free bases may have been to react with 5-phosphoribosylpyrophosphate and thereby to divert the metabolism of this material from the formation of glycinamide ribotides. It was suggested that IMP and the carboxamide ribotide may be important in regulating *de novo* synthesis by inhibiting reactions early in the scheme.

SYNTHESIS AND OCCURRENCE OF NUCLEOTIDES NOT RELATED TO NUCLEIC ACIDS

Synthesis and reactions of diphosphopyridine nucleotide.—Recent work has shown that the synthesis of DPN by mammalian enzymes proceeds by way of nicotinic acid-adenine dinucleotide (or desamido-DPN) as an intermediate. Preiss & Handler (65) and Langan & Shuster (66) have shown that shortly after injection of C^{14}-nicotinic acid into the rat or mouse the label was found in a compound identified as desamindo-DPN, but at later times only the DPN was significantly radioactive. In various enzyme systems from erythrocytes, yeast, or liver the following reactions account for DPN synthesis (67):

$$\text{nicotinic acid} + \text{5-phosphoribosylpyrophosphate} \rightarrow \text{nicotinic}$$
$$\text{acid nucleotide} + \text{pyrophosphate} \quad (6)$$
$$\text{nicotinic acid nucleotide} + \text{ATP} \rightleftharpoons \text{desamido DPN} + \text{pyrophosphate} \quad (7)$$
$$\text{desamido-DPN} + \text{glutamine} + \text{ATP} + H_2O \rightarrow \text{DPN} +$$
$$\text{glutamate} + \text{AMP} + \text{pyrophosphate} \quad (8)$$
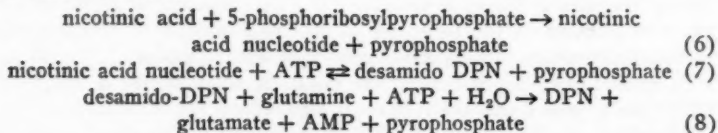
The enzyme of Reaction 6 is different from nicotinamide mononucleotide

pyrophosphorylase, but Reaction 7 is catalyzed by Kornberg's DPN pyrophosphorylase. Ammonia was as effective as glutamine in the final reaction, but at physiological pH glutamine is probably the most important donor. Azaserine inhibited this reaction as a competitor of glutamine. If azaserine were incubated with the enzyme in the presence of desamido-DPN, ATP, and magnesium ions, an irreversible combination between the enzyme and inhibitor took place.

In the presence of the enzyme DPNase the nicotinamide portion of DPN could be replaced by various pyridine and imidazole derivatives. Friedland *et al.* (68) have obtained the DPN and TPN analogues of the cytotoxic agent, 6-aminonicotinamide. 6-Amino-DPN inhibits DPN-dependent dehydrogenases. Anderson (69) reports that 3-benzoyl- and 3-acetylpyridine, pyridine-3-aldehyde, pyridine-3-aldoxime, and pyridine-3-hydrazide can participate in certain dehydrogenase systems, but that 3-amino-, 3-methyl-, and 3-acetamidopyridine are apparently inactive in the systems tested. A histidine-adenine dinucleotide is formed in good yield from DPN and histidine in the presence of DPNase, according to Alivasatos (70, 71). The analogous replacement of the nicotinamide of DPN by 5-amino-4-imidazolecarboxamide was previously described by Alivasatos & Woolley (72).

Flavin-adenine dinucleotide synthesis.—Enzymes catalyzing the synthesis of flavin-adenine dinucleotide from flavin mononucleotide and ATP have been found in a variety of animal tissues by DeLuca & Kaplan (73). The reaction is similar to the one described earlier by Schrecker & Kornberg (74) in a yeast enzyme system.

Nucleotide derivatives in the synthesis of polysaccharides.—The participation of uridine nucleotides in galactose, glucose, and glucuronic acid metabolism and of cytidine compounds in phosphatide synthesis has been reviewed recently (75, 76). It now appears likely that such nucleotide derivatives are involved in the synthesis of several biologically important polysaccharides. The net formation of chitin from uridine diphosphoacetylglucosamine by an enzyme from Neurospora has been described by Glaser & Brown (77). Glucose units from uridine diphosphoglucose can be added to cellodextrin primers in enzyme preparations from *Acetobacter xylinum* and may thereby be a precursor of cellulose (78). Leloir & Cardini (79) have reported that a soluble liver enzyme catalyzes the formation of glycogen from uridine diphosphoglucose in the presence of a macromolecular primer such as glycogen or starch. The relationship between nucleotide compounds and batcterial cell wall polymers was indicated by Park & Strominger (80), who found that uridine derivatives which accumulated during penicillin inhibition of *Staphylococcus aureus* were related in composition to material normally present in cell walls. Both the nucleotides and certain bacterial cell walls contain acetylmuramic acid (the 3-O-lactate ether of acetylglucosamine) or peptide derivatives thereof. Presumably the uridine nucleotides are "activated" forms of the acetylmuramic acid derivatives which participate in polymerization reactions to form the cell wall material. Strominger

(81) has recently reported the formation from uridine-diphosphoacetyl-glucosamine and phosphoenolpyruvate of a compound related to acetylmuramic acid. Armstrong *et al.* (82, 83) have recently discussed an analogous situation in *L. arabinosus, Bacillus subtilis,* and *S. aureus,* in the cell walls of which ribitol phosphate polymers were found. These polymers, called teichoic acids, also contain equivalent amounts of glucose and alanine. Although the nature of the linkage between these components has not been completely determined, the alanine residues are probably attached in the polymer in ester linkage and the glucose units through α-glycosidic bonds. Extracts of *L. arabinosus* contain two nucleotides which have been identified as cytidine diphosphoribitol and cytidine diphosphoglycerol by Baddiley and co-workers (82). The former of these compounds accumulates in *S. aureus* during penicillin inhibition. While the role of the glycerol-containing nucleotides is not known, it has been suggested that they may be involved in the formation of the protoplast membrane. Barry has found that a strain of *E. coli* contains colominic acid, a polymer of N-acetylneuramic acid (84). That uridine nucleotide derivatives of N-acetylneuraminic acid and certain peptide conjugates of this compound are produced by the bacteria suggests that these nucleotides may be important in the synthesis of the polymer (85). Cells of *Polyporus squamosus* contain a derivative of cytidylic acid which can be shown to yield a number of amino acids upon hydrolysis. The compound, designated as CMP-X, is normally ninhydrin negative. After mild acid hydrolysis, all the phosphorus and pyrimidine can be accounted for as 2′- and 3′-CMP (86).

New cytidine nucleotides.—Potter & Buettner-Janusch (87) reported the isolation from calf thymus of two compounds denoted as cytidine diphosphate-X and deoxycytidine diphosphate-X. Mild acid hydrolysis of the dinucleotides yielded cytidylic acid or deoxycytidylic acid as well as a phosphate ester of an unknown compound. The component X appeared to be a basic compound. Sugino and co-workers (88, 89) and Okazaki & Okazaki (90) have found that several natural materials including sea urchin eggs, microorganisms, and mammalian tissue contain deoxyribosidic compounds which do not support the growth of *Lactobacillus acidophilus* until the materials are hydrolyzed by crude preparations of snake venom enzymes. One such compound obtained from sea urchin eggs has been identified as deoxycytidine diphosphocholine. *L. acidophilus* contains deoxyribosidic compounds which are different from previously described substances and which must be hydrolyzed before they will stimulate growth of this organism. Acid soluble nucleotides similar to deoxycytidine diphosphocholine have been found in the Novikoff hepatoma by Schneider & Rotherham (91).

Interconversions of Purine and Pyrimidine Compounds

Deamination reactions.—The crystallization of muscle adenylic deaminase has previously been reported by Lee (92). Ito & Grisolia (93) have proposed a convenient method involving differential salt extraction for

obtaining partially purified adenylic deaminase from muscle free from myokinase. In contrast to the muscle enzyme, an adenylic deaminase isolated from brain by Mendicino & Muntz (94) has an absolute requirement for ATP. ATP could not be replaced by ADP, IDP, or ITP. The ATP could be recovered unchanged after the reaction and thus appears to play a catalytic role. P^{32}-labled AMP appeared to be directly converted to IMP, and deamination at the triphosphate level did not seem likely. Scarano (95) has described an enzyme isolated from unfertilized eggs and from embryos of the sea urchin which is capable of effecting the deamination of 5′-deoxycytidylic acid to 5′-deoxyuridylic acid. Laland et al. (96) have shown that enzymes from dried barley deaminate cytidine and deoxycytidine. Earlier reports that amino purines and pyrimidines could undergo transamination reactions with a α-ketoglutarate in the presence of enzymes from E. coli were reinvestigated by Schein & Brown (97). These workers could find no evidence for a transamination of this type from adenine, adenosine, cytosine, or cytidine in E. coli, rabbit liver, or rat liver enzyme preparations.

Interconversion of bases, nucleosides, and nucleotides.—Many purine and pyrimidine nucleotides may be formed directly by the enzymatic condensation between the free base and 5-phosphoribosylpyrophosphate, but in certain systems nucleotide synthesis may proceed via the intermediate formation of nucleosides.

Khorana et al. (98) have shown that the terminal pyrophosphate group of ATP is transferred as a unit to ribose-5-phosphate in the synthesis of 5-phosphoribosylpyrophosphate. The enzyme responsible for this reaction has been named 5-phosphoribose pyrophosphokinase. 5-Phosphoribosylpyrophosphate has now been synthesized chemically by Tener & Khorana (99) and the configuration of the biologically active material definitely shown to be alpha. In an earlier report, Remy et al. (26) had demonstrated that 5-phosphoribosylpyrophosphate, when formed enzymatically, is of the α configuration. The enzymes which condense phosphoribosylpyrophosphate with purine and pyrimidine compounds, the nucleotide pyrophosphorylases, have been described for the reaction of adenine and 5-aminoimidazolecarboxamide (100, 101), guanine, and hypoxanthine (100, 102), xanthine (39), orotic acid (103), and uracil (104). The enzymatic synthesis of nucleotides from purine analogues by similar reactions is discussed in the section on inhibitors of nucleotide metabolism.

Reichard & Sköld (105) have compared the metabolism of uracil in ascites tumor and in mouse and rat liver. Whereas ascites tumor can utilize uracil for nucleic acid synthesis, mouse and rat liver cannot. This is presumed to result from a lack of either nucleoside phosphorylase or uridine kinase in the latter two tissues and to the presence of these enzymes in adequate quantities in the tumor. It has been found by Canellakis (106), however, that uracil can be converted to the nucleotide and to nucleic acid in rat liver, provided that the concentration of the base is sufficiently high. Regenerating rat liver is more effective than normal liver in this respect.

Normal liver has a high level of enzymes for uracil degradation, and in order to demonstrate the utilization of uracil for nucleic acid synthesis enough of the compound must be added to insure that not all of it will be degraded. The catabolic capacity of regenerating liver is much lower so that uracil can be more efficiently utilized in anabolic processes (107).

Other reports concerning the transformations of purine or pyrimidine compounds at the nucleotide or nucleoside level include a discussion of the investigations of Razzell & Khorana (108), who have purified and described the properties of a pyrimidine deoxyriboside phosphorylase from *E. coli*. Roush & Betz (109) have isolated and purified a *trans*-N-deoxyribosylase from *Lactobacillus helveticus* which is capable of transferring the deoxyribosyl group of both purine and pyrimidine deoxyribonucleosides to guanine, adenine, hypoxanthine, 8-azaguanine, 6-mercaptopurine, thymine, uracil, and cytosine. A nucleoside phosphorylase reactive with uric acid riboside has been found in a variety of mammalian tissues (110) and is distinct from the enzyme of Kalckar, which phosphorylyzes inosine, adenosine, guanosine, etc.,

Nucleosides play an important role in carbohydrate metabolism of erythrocytes by making pentose available in a reactive form in the cells. The conversion of the pentose of various nucleosides to lactate has been studied by Lowy *et al.* (111). The initial action of a nucleoside phosphorylase upon the nucleoside to form a phosphorylated ribose derivative is important in the utilization of inosine, guanosine, and adenosine (112). An adenosine kinase has been purified from pigeon liver which phosphorylates adenosine, guanosine, and 5-aminoimidazolecarboxamide riboside to their corresponding nucleotides (113). An enzyme purified extensively from *Azotobacter vinelandii* catalyzes the phosphorylation of deoxycytidine-5′-phosphate or cytidine-5′-phosphate by ATP (114). Canellakis & Mantsavinos (107, 115) have found that soluble enzymes of regenerating rat liver in the presence of ATP can phosphorylate deoxyadenylic, deoxyguanylic, deoxycytidylic, and thymidylic acids to the triphosphate level but that the ability to phosphorylate thymidylic acid is very low in normal rat liver. Kornberg (116) has isolated and purified an enzyme from *E. coli* which catalyzes the reversible reaction between polyphosphate and ADP to form ATP. Studies on the kinetics of crystalline ATP transphosphorylases have been reported by Noda (117) and by Kuby & Mahowald (118).

Ahmed & Reis have described an activating effect of manganous ions on the 5′-nucleotidase reaction(119). The fact that 5′-nucleotidase is inhibited by nickelous ions but that other nonspecific phosphatases are not suggests a possible means of distinguishing between these types of enzymes.

The conversion of ribonucleotides and ribonucleosides to deoxy compounds.—The conversion of ribonucleotides to deoxy compounds has been studied in a variety of biological systems. Several authors have confirmed the original observation of Rose & Schweigert (120) that pyrimidine

nucleosides or nucleotides are reduced to their deoxyribose counterparts as intact units and without degradation, whereas the corresponding purine nucleotides undergo a partial loss or exchange of the ribose moiety before being similarly reduced. Edmonds (121) has examined deoxyribose formation in Ehrlich ascites cells. She has observed that randomly labeled C^{14}-adenosine undergoes exchange with nonradioactive deoxyadenosine to yield deoxyinosine labeled only in the hypoxanthine moiety. On the other hand, incubation of the ascites tumor cells with randomly labeled cytidine resulted in the formation of an acid-soluble fraction which upon hydrolysis yielded radioactive nucleosides and nucleotides of cytosine of both the ribose and deoxyribose varieties. Since the deoxy compounds contained C^{14} to an equal extent in both the pyrimidine and deoxyribose moieties, one can assume that a direct reduction of the ribose compound to a deoxyribose compound had taken place.

McNutt has shown that purine or pyrimidine mutants of *N. crassa* synthesize DNA and RNA from uniformly labeled adenosine (122) and cytidine (123), in which distribution of the isotope between base and sugar was essentially the same in either polymer, regardless of the precursor. Cytidine was incorporated into the polymers undiluted and as a unit. However, in the case of incubation with uniformly-labeled adenosine, the ribose and deoxyribose moieties of the isolated RNA and DNA contained about one-half the concentration of C^{14} as did the base. These experiments suggest that the loss of the ribosyl moiety may not be necessary for the reductive step itself but may represent a side reaction of adenosine prior to its direct reduction to deoxy compounds.

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At the enzymatic level, Reichard (125) has reported that minced chick embryo is capable of catalyzing the conversion of C^{14}-cytidine to DNA and RNA containing C^{14}-deoxycytidine and C^{14}-cytidine, respectively. When C^{14}-uridine was incubated with homogenates or a supernatant solution obtained from high speed centrifugation of the homogenates, a nucleotide was formed which was tentatively identified as deoxyuridylic acid. By far the best evidence, however, for the conversion of ribonucleotides to deoxyribonucleotides by enzyme preparations has been reported by Grossman & Hawkins (126) and Grossman (127). They have shown that soluble extracts of *S. typhimurium* LT-2 contain enzyme systems which catalyze the

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conversion of uridine and cytidine to deoxyuridine and deoxycytidine, respectively. The same extracts catalyze the conversion of cytidine-5′-phosphate to deoxycytidine-5′-phosphate. Loss of enzyme activity produced on dialysis may be fully restored by addition of either 1,3-dimercaptopropanol or 2,3-dimercaptopropanol (BAL).

CONVERSION OF PURINES TO OTHER HETEROCYCLIC COMPOUNDS

Previously, McNutt has shown that adenine labeled in the pyrimidino portion of its purine ring contributes specifically to the pyrimidino ring of the isoalloxazine derivative, riboflavin (128). Al-Khalidi (129) has reported that guanine-2-C^{14}, but not guanine-8-C^{14}, is an isotopic precursor of riboflavin in *Eremothecium ashbyii*, an observation which supports the earlier view that carbon 8 of a purine is replaced by a two-carbon unit, of some type, in riboflavin formation. There is suggestive evidence from this work that guanine is a more direct precursor of riboflavin than is adenine, since guanine is not appreciably converted to adenine compounds in this organism. In agreement with this result Brown *et al.* (130) found, in studies on the stimulation of riboflavin production by the same organism, that guanine, xanthine, adenine, hypoxanthine, and uric acid were decreasingly effective in that order. McNutt & Forrest (131, 132) have isolated a radioactive pteridine derivative, possibly related to riboflavin biosynthesis, which is formed in small amounts by *E. ashbyii* metabolizing C^{14}-adenine. The compound, probably a 2,4-dihydroxypteridine, did not seem to be a precursor or a product of riboflavin metabolism.

A metabolic relationship between purines and the pteridines, the folic acid vitamins, has been demonstrated in *Gaffkya homari* (133). This microorganism has a unique nutritional requirement for either a purine or a pteridine derivative related to folic acid. Since folic compounds are formed by this organism if purines are supplied, it is proposed that purines are precursors of these pteridine compounds. The similarity between the precursors of the pteridines and those of the purines has previously been pointed out (134).

Further studies on the formation of imidazoleglycerol phosphate from ATP, ribose-5-phosphate, and glutamine by bacterial enzymes have been reported by Moyed (135). The results indicate a multiple-step process. One of three enzyme fractions which have been obtained catalyzed the reaction between ATP and ribose-5-phosphate to yield 5-phosphoribosylpyrophosphate and AMP. In a second step, AMP and phosphoribosylpyrophosphate reacted to form an intermediate with a phosphoribosyl substituent at the 1-position of AMP. In the presence of the third enzyme fraction and glutamine the intermediate was converted to aminoimidazolecarboxamide ribotide and imidazoleglycerol phosphate, possibly in a two-step reaction. These reactions are essential for histidine formation, and histidine exerts a feedback inhibition on the formation of the three enzymes.

Mode of Action of Inhibitors of Nucleotide Metabolism

Several new 5-fluoropyrimidine derivatives have been synthesized by Duschinsky and co-workers (136 to 138). It has been found primarily through the work of Heidelberger's group (139 to 143) and by Eidinoff *et al.* (144) that 5-fluorouracil and related compounds inhibit mammalian tumors by interfering in a rather complex way with both RNA and DNA synthesis. The fluorinated pyrimidines inhibit the incorporation of formate, orotic acid, and pyrimidines into thymine of DNA in liver, spleen, and the Ehrlich tumor *in vivo*. The incorporation of uracil and orotic acid into uracil of RNA is also blocked. The riboside and deoxyriboside of fluorouracil were more potent than the base, but none of these compounds inhibited DNA thymine formation from thymidine (139). Fluorouracil and fluororotic acid were converted to acid-soluble fluorouridine phosphates and incorporated into RNA but not into DNA in normal tissues and tumors of the mouse. Fluorouracil was selectively taken up by tumor tissues *in vivo* (140). Similar results were obtained by Bosch, Harbers & Heidelberger with Ehrlich cells *in vitro* (141). These workers have concluded that in ascites cells the fluorinated pyrimidines block the methylation of deoxyuridylic acid to thymidylic acid and the incorporation of precursors into RNA uracil by interfering with uracil metabolism (141, 142, 143). According to Shapira & Winzler (145) fluorouracil also inhibits the formation of DNA thymine from C^{14}-formate and other precursors in granulocytic leukemia leucocytes.

Cohen *et al.* (146) and Scheiner & Duschinsky (147) have shown that fluorouracil and fluorouridine affect both thymine and uracil metabolism in *E. coli* in a manner analogous to the situation in tumor tissue. Fluorodeoxyuridine was the most potent bacterial inhibitor of this group. Cohen *et al.* found that fluorodeoxyuridine, by conversion to fluorodeoxyuridylic acid, produced a condition of thymine deficiency, unbalanced growth through inability to synthesize DNA, and death similar to that which occurs in the thymineless mutant 15 T⁻ of *E. coli*. This inhibitory effect on thymine formation is of much greater significance to the survival of the bacteria than is the effect on uracil incorporation into RNA. The action of the fluorouracil compounds on thymine synthesis has been shown to consist of the irreversible inhibition of the enzyme, thymidine synthetase, by fluorodeoxyuridylic acid (Reaction 9).

$$\text{deoxyridylic acid} + \text{HCHO} + 2\text{H} \xrightarrow{\text{THFA}} \text{thymidylic acid} \qquad (9)$$
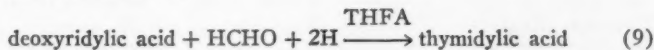
Enzymatic mechanisms are available in the cell for formation of fluorodeoxyuridine from fluorouracil and its phosphorylation to fluorodeoxyuridylic acid.

The mechanism of fluorouridylic acid formation and of the inhibition of uracil metabolism by fluorouracil has been clarified by Sköld in ascites tumor systems (148). In contrast to most mammalian tissues, Ehrlich ascites

cells can utilize uracil for RNA synthesis since they possess a nucleoside phosphoryase and nucleoside kinase for uridine. Fluorouracil inhibits uridine formation from uracil by its action on the nucleoside phosphorylase but does not affect uridine kinase. These two enzymes can also convert fluorouracil to fluorouridylic acid. Since the fluorouracil compounds can be incorporated into RNA in mammalian and tumor tissues, Sköld suggests that the formation of an abnormal RNA might be a third point of action of these compounds. The formation of tobacco mosaic virus by infected leaves is inhibited by fluorouracil (148a). This inhibition, which seems to derange the metabolism of the host as well as that of the virus, cannot be reversed by thymidine. While fluorouracil causes a net inhibition in the formation of tobacco mosaic virus, Gordon & Staehelin (149) find that the compound can replace about one-third of the uracil in viral RNA and that the virus so formed is fully virulent. In *E. coli,* however, there is probably little or no utilization of fluorouracil compounds for RNA synthesis. Horowitz, Saukkonen & Chargaff (150) have demonstrated that in a uracil auxotroph of *E. coli* fluorouracil can partially replace uracil for amino acid incorporation into proteins, but that no RNA or DNA synthesis takes place in the presence of fluorouracil. While net protein synthesis will take place, the cells cannot be induced to form the adaptive enzyme, β-galactosidase, in the presence of fluorouracil.

In Ehrlich ascites tumor *in vitro,* 6-uracil methylsulfone has an effect similar to fluorouracil in that the methylation step of thymine synthesis is blocked. On the other hand, there seems to be no inhibition of RNA pyrimidine formation from orotic acid (151).

The incorporation of uracil into RNA in rat hepatoma (152) is depressed by 2-thiouracil. The work of Amos (153) suggests that the bacteriostatic action of thiouracil may result from the formation of an altered RNA. Thiouracil can replace up to 20 per cent of the uracil of the RNA in *E. coli,* at which point net RNA synthesis and cell division cease. DNA and protein synthesis, as well as RNA turnover, may continue for some time, but the cells become morphologically altered and nonviable. It was suggested that the incorporation of thiouracil into RNA results in the formation of nonfunctional protein. The inhibitory effect of thiouracil on the growth of *E. coli* is antagonized by potassium ions. Amos *et al.* (154) have reported that potassium specifically inhibits the enzyme system in the cells which converts thiouracil to its nucleotide derivative, while the corresponding conversion of uracil is not affected. Thiouridylic acid would therefore seem to be the active agent in this bacterial inhibition. In the presence of 2-thiouracil, RNA synthesis during influenza virus infection is depressed and part of the virus formed under these conditions is nonviable. This inhibition is reversed by uracil (155).

6-Azauracil is an inhibitor of the multiplication of microorganisms and the growth of certain tumors. Sorm and co-workers (156, 157, 158) have shown that the multiplication of *E. coli* cells is more strongly inhibited by

the free base while Ehrlich tumor cells are more sensitive to the riboside. In the presence of 6-azauracil *E. coli* cells accumulate the riboside in the medium. This process may be used for the preparation of the riboside in quantity. Under these conditions of growth the cells also excrete orotic acid, orotidylic acid, uracil, uridylic acid, and hypoxanthine (156, 159). In cancerous tissue the riboside is converted largely to the ribotide (157). The evidence of Melnick *et al.* (152) and Šorm *et al.* would suggest that the conversion of orotic acid and uracil to RNA pyrimidines is inhibited by these analogues in tumors. From studies on *E. coli,* Takagi & Otsuji (160) suggest that 6-azauracil may interfere with cell wall synthesis from uridine compounds since RNA synthesis continues in the presence of azauracil and N-acetylglucosamine compounds accumulate.

Whereas fluorouracil appears to be a uracil antimetabolite, the 5-bromo-, 5-chloro-, and 5-iodouracils can be considered thymine analogues insofar as they may be incorporated into DNA in the place of thymine. Zamenhof *et al.* (161) have shown that 5-bromouracil can replace up to 50 per cent of the thymine in DNA in thymine requiring strains of *E. coli.* The chloro- and iodouracils are also incorporated into the DNA of *E. coli* while the bromo- and iodo-derivatives are found in the DNA of T2 and T5 phage, according to Dunn & Smith (162). A portion of the phage containing these analogues was nonviable. While thymine could overcome the inhibition produced by 5-bromo- and 5-iodouracil, both thymine and uracil were required to reverse the action of chlorouracil. Bromouracil has a mutagenic effect on phage (163). Benzer & Freese (164) have now shown that the type of mutations produced by the analogue are different from those occurring spontaneously. 5-Bromodeoxyuridine inhibits the methylation step in thymidylic acid formation in various mammalian and tumor tissues (165). Hakala finds that bromodeoxyuridine will replace thymidine in the growth of HeLa cells in the presence of 4-aminomethylpteroylglutamic acid, but that the cells formed under these conditions are morphologically altered from the normal (166).

Purine analogues.—8-Azaguanine inhibits the growth of certain microorganisms and this inhibition is reversed by normal purines. This compound can replace up to 40 per cent of the guanine in RNA of *Bacillus cereus* (167). The unnatural RNA constituent seems to be selectively lost by metabolic turnover in the presence of guanine compounds, probably because a large portion of the incorporated azaguanine is present at the ends of polynucleotide chains (168, 169). Chantrenne & Devreux (167) and Mandel & Markham (168, 169) find that 8-azaguanine stimulates RNA synthesis in *B. cereus.* The amount of adenine, cytosine, and uracil nucleotides incorporated is increased in proportion to the amount of 8-azaguanine utilized, while the amount of guanine incorporated into RNA is constant (169). It is proposed that azaguanine replaces guanine only in the excess RNA formed. Mandel (170) finds that azaguanine inhibits growth and total protein synthesis equally, but selectively suppresses the uptake of S^{35}-methionine

and cystine into protein. The corresponding uptake of radioactivity from acetate or glutamate into protein, however, is not selectively suppressed. On the other hand, Chantrenne & Devreux (167) have observed a marked inhibition of both the net synthesis of protein and the uptake of methionine, valine, leucine, and phenylalanine into protein. The formation of DNA and cell wall material is not affected during the early stages of this inhibition but eventually, if the azaguanine concentration is sufficiently high to prevent replacement by guanine in RNA, then DNA, RNA, protein, and cell wall syntheses cease. The latter workers liken the effect of azaguanine to that described for chloramphenicol. Under these conditions, it is believed, an unstable RNA is formed and protein synthesis is inhibited. The unstable RNA is degraded upon removal or exhaustion of the inhibitor.

The mechanisms of action of other structural analogues of purines are not clear as yet. Among those which have been described as inhibitory in bacterial or tumor systems are 6-thioguanine (178, 179), 6-mercaptopurine (166, 178), 1-deazaguanine (180), 6-methylpurine (181), and purine (166). Hampton et al. (171) have synthesized the 6-mercapto analogue of adenylo-succinic acid and 6-mercaptopurine nucleotide and find that these compounds inhibit the cleavage of adenylosuccinic acid to adenylic acid by the enzyme adenylosuccinase. The former of these compounds has been found to block the cleavage of 5-aminoimidazole-N-succinocarboxamide ribotide also cata-lyzed by this enzyme (172). The inhibition of growth of HeLa cells by 6-mercaptopurine is reversed by adenine and hypoxanthine but not by aminoimidazolecarboxamide. Hakala (166) interprets this finding to indicate that the mercapto compound interferes with purine biosynthesis and not with the interconversion or utilization of the purine. In contrast, inhibition of the cells by purine can be antagonized by adenine but not by hypoxanthine compounds, a fact which suggests specific interference with adenine synthesis.

Many of the analogue inhibitors of purine and pyrimidine metabolism are biologically active in the form of their nucleotide derivatives, and enzymatic mechanisms are normally present in cells which can convert these bases to the nucleotide. Way & Parks (173) find that enzymes of hog liver can catalyze the reaction of 8-azaguanine, 2,6-diaminopurine, 6-mercapto-purine and several pyrazolo (3,4-d) pyrimidines with phosphoribosylphos-phate to form ribotides of the analogues. Lukens & Herrington (102) had found earlier that a nucleotide pyrophosphorylase reactive with hypoxan-thine and guanine would also form 6-mercaptopurine ribotide from the free base. The absence of a nucleotide phosphorylase appears to be important in the acquisition of resistance to purine analogues by bacteria and tumors. Thus Brockman et al. (174, 175) find that the acquisition of resistance to 6-mercaptopurine and azaguanine in Streptococcus faecalis and to azagua-nine in a strain of leukemic leucocytes (L 1210) results in the inability of the cells to convert these compounds to the ribotide. In the resistant strains, hypoxanthine and guanine cannot be utilized either, but xanthine can. Certain 6-mercaptopurine-resistant strains of S. faecalis are found to behave

similarly by Balis *et al.* (176, 177). In others, another mechanism of drug resistance seems operative but is as yet not understood.

Inhibitors of purine synthesis de novo.—Studies with the antimetabolites azaserine (O-diazoacetyl-L-serine) and 6-diazo-5-oxo-L-norleucine in isolated enzyme systems had shown them to act antagonistically to glutamine in several enzymatic reactions of the latter compound. These compounds inhibit purine synthesis by combining with and inactivating the enzyme responsible for the conversion of formylglycinamide ribotide to formylglycinamidine ribotide (182). Inhibitions of a lower order of magnitude are achieved by azaserine in other reactions of glutamine, in which cases the analogue acts competively with glutamine. These reactions include the formation of phosphoribosylamine from 5-phosphoribosylpyrophosphate (27, 183), the amination of xanthosine-5'-phosphate to guanosine-5'-phosphate (41), and the amidation of desamidodiphosphopyridine nucleotide to DPN (67).

Greenlees & LePage (184) had reported that azaserine produced an accumulation of glycinamide ribotide rather than formylglycinamide ribotide when the inhibitor was given to rats with Ehrlich ascites tumors. It was proposed that in tumor the glutamine analogue was blocking the formylation of glycinamide ribotide rather than the reaction of formylglycinamide ribotide, which is inhibited in pigeon liver extracts and in *E. coli*. Upon reanalysis of the products of the incubation Moore & LePage (185) now find, however, that both tumor and normal tissue of the mouse respond similarly to the above-mentioned systems in that the compound which accumulates is actually formylglycinamide ribotide rather than glycinamide ribotide. A small amount of an unidentified radioactive compound was found in liver, kidney, and intestine of animals administered glycine-2-C^{14} and azaserine. At low concentration 6-diazo-5-oxo-L-norleucine had effects similar to those of azaserine, but at higher concentrations of this more potent inhibitor formylglycinamide ribotide was no longer formed. It seems possible that the reaction between glutamine and 5-phosphoribosylpyrophosphate to form phosphoribosylamine (see Figure 1) may be inhibited by higher concentrations of 6-diazo-5-oxo-L-norleucine and that the formation of the acyclic ribotides may be prevented.

Sartorelli & LePage (179) have tested the combined effect of azaserine and 6-thioguanine for their inhibition of several tumors. Tarnowski & Stock (186) had previously shown the synergistic action of azaserine, an inhibitor of *de novo* purine synthesis, in combination with thioguanine, an inhibitor of the metabolism of preformed purines. The Ehrlich ascites, TA3 carcinoma, and Sarcoma 180 tumors were sensitive to this combination, but some other tumors tested were not (179).

Anderson *et al.* (187) have shown that the acquisition of azaserine resistance in a line of neoplasm 70429 does not alter the pathway of purine biosynthesis in these cells since purine synthesis in cell-free extracts of both sensitive and resistant lines is blocked by azaserine. The resistant lines may

have acquired a permeability barrier to azaserine. Sartorelli & LePage find that a resistant line of the TA3 carcinoma, while initially inhibited by azaserine, recovers the ability to form purines *de novo* more rapidly than does the sensitive line (188).

Eidinoff *et al.* (11) have reported that 6-diazo-5-oxo-L-norleucine depresses the formation of nucleic acid cytosine, but not that of uracil or thymine, from ureidosuccinic acid and orotic acid in liver, intestine and tumors of rats. The inhibitor does not block cytidylic acid incorporation into RNA but does block the conversion of uridylic acid to RNA-cytosine. The fact that glutamine could partially overcome this inhibition suggested that this amino acid was involved in cytidylic acid formation. This conclusion has now been confirmed by the work of Kammen & Hurlbert (13, 14).

ENZYMATIC SYNTHESIS OF POLYNUCLEOTIDES

Deoxyribonucleic Acid.—Kornberg and associates (189, 190) have now reported in detail experiments concerned with the enzymatic synthesis of DNA. They have isolated and purified four-thousand-fold an enzyme from *E. coli* which carries out the synthesis of DNA from the deoxynucleoside triphosphates of adenine, guanine, thymine and cytosine with the liberation of inorganic pyrophosphate. Magnesium ions as well as a small amount of undegraded DNA primer, which may be obtained from a variety of sources, are essential components of the reaction. The enzymatic system has now been developed so that the amount of DNA synthesized is approximately 10 to 20 times greater than that of the added primer. The molecular weight of the newly synthesized material is approximately 5 million (191). On the basis of physical chemical measurements, this product cannot be distinguished from primer DNA.

The existence of the incorporated nucleotides in the $3':5'$-phosphodiester bridges was demonstrated by degrading C^{14}-labeled DNA synthesized from C^{14}-labeled thymidine triphosphate and the other nonradioactive nucleotides with crystalline pancreatic deoxyribonuclease. The hydrolysis of DNA by this enzyme liberates nucleoside $5'$-phosphate or polynucleotides with $5'$-phosphate groups. Dinucleotides of thymidylic acid and of thymidylic acid and cytidylic acid were isolated containing C^{14}. When the experiment was repeated with P^{32}-labeled deoxynucleotides of adenine, guanine, and cytosine but with the omission of thymidine triphosphate, a small amount of P^{32}-DNA was formed which was again degraded with pancreatic deoxyribonuclease. The thymidylate dinucleotide was devoid of radioactivity whereas dinucleotides containing any other of the three nucleotides were labeled with P^{32}.

Possibly the most conclusive evidence for the reaction of the nucleoside triphosphate with DNA to form $3':5'$-phosphodiester bridges comes from experiments in which a single P^{32}-labeled nucleoside triphosphate was reacted in the absence of the other three nucleotides (192). Under these conditions the incorporation of the radioactive nucleotide into DNA was

greatly reduced in comparison to its incorporation in the presence of the other three substrates. Reaction of the radioactive nucleoside triphosphate presumably takes place primarily with the end groups of the primer DNA. This radioactive product was degraded by the combined action of micrococcal deoxyribonuclease and spleen phosphodiesterase to the deoxynucleoside 3'-phosphates. The radioactive phosphorus, originally present in the one nucleoside 5'-triphosphate substrate, was found in all of the nucleoside 3'-phosphates from the degraded DNA. These results indicate that the radioactive nucleoside triphosphate was added to the DNA chains at the deoxynucleoside end. Any one of the four nucleosides could occupy this terminal position. A significant portion of the added nucleotides in turn become sites for further addition. However, all or most of the radioactive nucleotides are near the end of the chain. This was demonstrated by treating the radioactive nucleic acid with snake venom diesterase, which is believed to cleave the nucleic acid chain sequentially. The small number of nucleotides released from the terminal positions of the chains contained a major portion of the radioactivity.

The pyrophosphorolysis of DNA, as measured by the incorporation of radioactive pyrophosphate into nucleoside triphosphates in the presence of polymer, may be demonstrated, but only under very special conditions (190). The incubation of DNA, pyrophosphate, and enzyme does not result in a significant cleavage of the polymer. Only in the presence of one or more of the nucleoside triphosphates does the reaction assume a quantitative significance. An optimal rate of pyrophosphorolysis is obtained in the presence of all four nucleotides. However, a significant reaction (40 per cent of the optimal) is obtained if only one nucleotide is present. It will be recalled that, in contrast, the omission of any one of the nucleoside triphosphates abolished the polymerization of the nucleoside triphosphates to DNA almost completely. The function of the nucleoside triphosphates in the pyrophosphorolysis of DNA is not at present understood.

The ability of certain analogues to replace the naturally occurring bases of DNA as substrates for the synthesis (193) has been studied. To varying extents the nucleoside triphosphates of uracil and 5-bromouracil specifically replace thymine; 5-methyl- and 5-bromocytosine replace cytosine; and hypoxanthine replaces guanine. Deoxyxanthosine triphosphate was not utilized in deoxyribonucleic acid synthesis. The specific replacement of the natural bases by certain analogues has been suggested as additional support for the base-pairing relationships in the double helix proposed by Watson and Crick for the structure of DNA.

Although an enzyme is available for the phosphorylation of 5-bromodeoxyuridine monophosphate to its triphosphate, the corresponding phosphorylation of deoxyuridylate does not occur. The inability of uracil to be incorporated into DNA does not reflect the inability of the enzyme to utilize the deoxyuridine triphosphate but rather the lack of a proper phosphorylating enzyme. Apparently there is a separate and specific kinase for the phos-

phorylation of the naturally occurring deoxynucleotides to the triphosphates. Work with cruder preparations of the polymerase was hampered by the presence of an enzyme, now separated and isolated, which destroys deoxyguanosine triphosphate by its cleavage to deoxyguanosine and tripolyphosphate (194).

The synthesis of DNA from the deoxynucleoside triphosphates has now been demonstrated with soluble enzyme preparations from animal tissues and tumors. Bollum (195) and Bollum & Potter (196) have found significant amounts of the enzyme in regenerating liver, thymus, small intestine, and spleen of the rat. Smaller amounts of enzyme were demonstrated in testes, kidney, normal liver, brain, heart, pancreas, and skeletal muscle. The enzyme was also found in relatively large amounts in extracts of Flexner-Jobling carcinoma and Walker 256 carcinoma. Harford & Kornberg (197) have shown the polymerase to be present in extracts of HeLa cells. Significant but smaller amounts were found in extracts of mouse spleen, lymph nodes of an immunized guinea pig, leucocytes of a leukemic patient, and calf thymus gland. Mantsavinos & Canellakis (198, 199) have also reported the preparation of a soluble enzyme system from rat liver which utilized the four deoxynucleotides for DNA synthesis and required added DNA for optimal activity. Davidson *et al.* (200) have described experiments in which 4-T-thymidine was incorporated into DNA by extracts of Ehrlich ascites cells.

A curious enzymatic reaction has been discovered by Hurwitz (201) in which radioactive cytidine triphosphate was utilized for polymer synthesis when incubated with other nucleoside triphosphates, Mn^{++} or Mg^{++}, DNA, and a partially purified enzyme isolated from *E. coli*. DNA, an essential component of the reaction, could not be replaced by tobacco mosaic virus ribonucleic acid. The radioactive product of the reaction may be digested by deoxyribonuclease. Of the acid-soluble nucleotides, only cytidine ribonucleotide was radioactive. The corresponding deoxyribonucleotide was inactive. The enzyme isolated by Hurwitz is different from the DNA polymerase described by Kornberg and his associates in that the latter enzyme is inactive in the presence of Mn^{++} and does not incorporate ribonucleotides into DNA.

Polyribonucleotides.—The rapidly developing field of the biosynthesis of the polyribonucleotides has been excellently documented in Volume 27 of these Reviews (2). Since much of this material has anticipated articles which have appeared within the present review period, the reader should refer to the former review for a more comprehensive discussion of the synthetic polynucleotides.

It has been found that in the polymerization of ADP by polynucleotide phosphorylase isolated and highly purified from *A. vinelandii*, a long lag period occurs which may be overcome specifically by the addition of polyadenylic acid (202). The polymerization of the other nucleoside diphosphates also requires the addition of specific primers (203, 204). However, the polymerization of either ADP or UDP may be initiated by the addition

of oligonucleotides as primers such as the di-, tri-, and tetranucleotides of adenine. Oligonucleotides with a phosphomonoester group at the terminal C-5' position readily underwent phosphorolysis, although the terminal phosphate group was not necessary for action of the enzyme. On the other hand, if the phosphomonester group were at the terminal C-3' position, the compounds were resistant to enzymic attack (205).

The synthesis of a polymer containing all four nucleotides is accomplished with either RNA or polycytidylic acid as primer. By the use of a P^{32}-labeled nucleotide, it may be shown that each of the nucleotides in the presence of the other three is incorporated randomly into the integral structure of the polynucleotide strand (206, 207, 208). Digestion of this synthetic RNA with ribonuclease results in the formation of radioactive 2'- and 3'phosphates of all four nucleosides.

Beers (209) has reported in detail the relative effects of salt, magnesium, substrate, and hydrogen-ion concentrations on the rate of polymerization of adenosine diphosphate to polyadenylic acid catalyzed by polynucleotide phosphorylase obtained from *Micrococcus lysodeikticus*. In contrast to the enzyme from *A. vinelandii*, polynucleotide phosphorylase from *M. lysodeikticus* requires a high concentration of KCl (or other salt) for optimal activity. The presence of salt is also related to the interaction of magnesium ions in the system. Acridine orange and polyadenylic acid form complexes the composition of which depends on the concentration of polynucleotide (210, 211). The combination of dye with polynucleotide is followed by marked changes in the spectrum in the visible region. The formation of one complex at low polynucleotide concentrations probably involves the interaction of the dye with the amino groups of the polymer, since reaction of the polymer with formaldehyde prior to reaction with dye results in the loss of its ability to bind the dye. At higher concentration of polynucleotide a second complex is formed in which the dye may be bound to the phosphate groups of the nucleotides. The interaction of polynucleotide with dye at this level is influenced by the concentration of magnesium ions, salt, and substrate. Presumably the dye either inhibits or stimulates polynucleotide synthesis from ADP by interaction with the polynucleotide primers which are formed by the enzyme and which are necessary for further reaction.

The polynucleotide phosphorylase derived from *M. lysodeikticus* differs from that from *A. vinelandii* in that, in addition to requiring a high concentration of ions for activity, no primer has been shown to be required for initiation of the reaction. Another possible difference may be that each of the four dinucleotides are polymerized by a single enzyme in *A. vinelandii*, whereas individual enzymes may be responsible for the polymerization of ADP and CDP in *M. lysodeikticus* (212, 213). The thesis that polynucleotide synthesis from the different nucleoside diphosphates in *M. lysodeikticus* is accomplished by separate enzymes must be regarded with some reservation until further evidence is available. The possibility exists that the results obtained by Olmsted (212, 213) can be accounted for by the action of

only one enzyme whose reactivity to the individual nucleoside diphosphates is controlled by complicated variations of pH, salt concentration, or the presence of specific inhibitors.

Hendley & Beers (214) have studied the phosphorolysis of polyadenylic acid by a purified preparation of polynucleotide phosphorylase from *M. lysodeikticus* freed from myokinase and phosphatase. Like the polymerization, the phosphorolysis is dependent upon the presence of magnesium ions and upon a high ionic strength. A high concentration of magnesium ions inhibits the polymerization reaction but not the phosphorolysis. The equilibrium ratio of ADP to orthophosphate is independent of the polyadenylic acid concentration. This equilibrium ratio may be increased fourfold by increasing the concentration of magnesium ions and by lowering the ionic strength.

A third type of polynucleotide phosphorylase has been isolated from yeast by Grunberg-Manago and her collaborators (215, 216) and purified thirtyfold. An assay involving the exchange of radioactive phosphate with the terminal phosphate of ADP was employed. Magnesium ions, which are required for polymer synthesis from IDP, CDP, UDP, and GDP, are not required for the exchange reaction. The product contains the synthesized polynucleotide complexed with protein. The polynucleotide, when separated from the protein, differs from the polyadenylic acid synthesized by bacterial polynucleotide phosphorylase in its ultraviolet absorption spectra as a function of pH, its lower reactivity with formaldehyde, its lack of interaction with polyuridylic acid, its markedly greater anionic electrophoretic mobility and its acid, alkali, and enzymatic hydrolysis products. The polymer contains phosphate groups other than those involved in the internucleotide linkages.

The structure and interaction of the various types of polynucleotides of adenine, cytidine, uridine, and hypoxanthine have received further attention during the past year (217 to 226). Following the observation that polyadenylic acid exists as a two-stranded structure (217), Rich (218) has now shown that polyinosinic acid has a three-stranded configuration. In addition other species have been discovered, e.g., a two-stranded polymer between polyinosinic acid and polyadenylic acid and a three-stranded structure which includes two polyinosinic acid chains and one polyadenylic acid chain (219). A complex of unknown structure is formed when polyinosinic acid and polycytidylic acid are mixed in a 1:1 ratio (220). A two-stranded complex between polyadenylic acid and polyuridylic acid has been described which will rearrange to form a three-stranded variety when the mole fraction of polyadenylic and polyuridylic acid is 1:2 (221, 222). The formation of these complexes may be followed by the hypochromic effect (223) exhibited when the combination of the two species takes place. The complexing phenomenon is affected by temperature, concentration of salt, magnesium and hydrogen ions (223, 224). From theoretical and experimental considerations it is believed that the alignment of the two strands takes place in such a

way that few gaps or uncomplemented spaces are found in the structure (225). Evidence is also available that the formation of the complexes is a fully reversible process (221).

The important and yet unanswered question to investigators in this field is whether the product formed in the presence of all four nucleoside triphosphates is identical with naturally occurring ribonucleic acid and whether the polynucleotide phosphorylase system represents the major pathway of synthesis of ribonucleic acid in animal and microbial systems.

NUCLEIC ACID SYNTHESIS IN ANIMAL SYSTEMS

Cell-free systems which incorporate nucleotides into RNA.—While soluble enzymes have been obtained from bacterial and mammalian cells which catalyze the synthesis of DNA and from bacteria which form RNA-like polymers, little is known of the mechanism of RNA formation in animal tissues. When radioactive adenine nucleotides (in particular, C^{14}-labeled ATP) are incubated with homogenates of liver (227) or Ehrlich ascites cells (228), the RNA becomes labeled to some extent in the inner nucleotides but predominantly in the terminal nucleotide. Upon treatment of the radioactive RNA with alkali, radioactive 2'- and 3'-phosphates as well as adenosine are found in the hydrolysate. The incorporation of radioactivity into the internal and into the terminal nucleotides involves two different enzyme systems. Herbert (227) has demonstrated that the enzymes responsible for the appearance of adenine nucleotides as the inner nucleotide moieties of RNA are present in the particulate fractions of tissue homogenates (i.e., the nuclei, microsomes, and mitochondria). On the other hand, an enzyme system present in the soluble portion of the tissue homogenate is responsible for the attachment of ATP as a terminal nucleotide to a specific type of RNA. This enzyme system, which was first reported by Zamecnik et al. (229), has now been purified by Herbert approximately eightyfold (230). It has been shown by Hecht et al. (231, 232) and confirmed by Herbert that C^{14}-ATP reacts with cytidylic acid end groups of the low molecular weight, soluble RNA of rat liver. When these cytidylic end groups are missing, they must be introduced through the reaction of the RNA with CTP before the adenylic acid groups can be attached. A product of these reactions is pyrophosphate. Terminal adenylate groups of the soluble RNA fraction may be cleaved by reaction with pyrophosphate to form ATP. Hecht et al. (231) have concluded that the end unit containing cytosine and adenine nucleotides provides a functional grouping in the soluble RNA which is required for its action as a carrier of activated amino acids in protein synthesis. This special type of nucleotide incorporation is a process probably not related to the general synthesis of RNA.

Edmonds & Abrams (228) have prepared soluble extracts of Ehrlich ascites cells which catalyze the incorporation of C^{14}-ATP into the terminal and inner nucleotide positions of RNA. They have presented evidence which indicates that ATP rather than ADP or AMP is the true reactant

in these systems. The RNA-dependent incorporation of radioactive pyrophosphate into nucleoside triphosphate in extracts of embryonic chicken livers described by Chung (233) and by Chung & Mahler (234) probably represents another example of the pyrophosphorolysis of end groups of RNA to yield nucleoside triphosphates. Thus, although the definitive enzymatic mechanisms for RNA synthesis have not been established in animal tissues, there is now growing evidence that nucleoside triphosphates may be direct precursors of nucleic acid nucleotides.

Cellular site of RNA synthesis.—Previous work has suggested that the nucleus may actually be the site of cytoplasmic RNA synthesis and a recent report of Scholtissek *et al.* (235) lends further support to this view. If labeled orotic acid were administered to rats in a single injection, the specific radioactivity of the RNA of liver nuclei reached a peak in 1 to 3 hr. while that of the cytoplasmic RNA slowly increased over a 16 hr. period. Radioactivity was lost from the nuclear RNA in a two-stage process. Rat liver nuclei were labeled in vivo by injection of labeled orotic acid. If the nuclei were isolated shortly after injection and then incubated with unlabeled liver cytoplasmic fractions, almost all of the radioactivity of the nuclear RNA was transferred to the cytoplasm. However, if the nuclei were isolated several hours after administration of the label, they retained most of their radioactivity in the subsequent *in vitro* incubation. Logan (236) has studied the incorporation of adenine into two separable fractions of RNA in isolated calf thymus nuclei. Both the adenine and guanine of nucleic acid were labeled. This observation suggests that the incorporation measured represented actual synthesis and not just end group attachment. RNA I, which was extracted at a low salt concentration, was labeled much more slowly than was RNA II, a component which was extracted at higher ionic strengths. In these short-term experiments (3 hr.), DNA-adenine was only slightly labeled. Isolated nuclei from rabbit appendix rapidly incorporated labeled formate into their protein, RNA, and DNA. The results indicate a metabolic heterogeneity of the RNA (237). Osawa *et al.* (238) and Hotta & Osawa (239) have also found this metabolic heterogeneity in the nuclear RNA of calf thymus as well as in several other mammalian tissues and have shown that, in general, RNA I and cytoplasmic (microsomal) RNA were indistinguishable in composition and in certain physical properties. The nuclear RNA with the high turnover rate was markedly different from each of these. While it would be tempting to postulate on the basis of their similarities that the nuclear RNA I is the precursor of microsomal RNA, these workers conclude that RNA II but not RNA I was synthesized at a sufficient rate to serve as such a precursor.

Ability of certain tissues to synthesize purines de novo.—It has been observed that the nucleic acid purines of several tissues become labeled after the administration of radioactive formate or glycine to intact animals. However, if many of the same tissues are grown in culture in the presence of labeled formate the labeling of the nucleic acid purines is markedly de-

pressed. Of several normal and malignant tissues which have been studied, liver is notable for its ability to carry out *de novo* purine synthesis actively *in vitro* as well as *in vivo* (240). Cell-free preparations from intestinal mucosa (237, 241) and from thymus (242) have also been shown to synthesize purines readily *de novo*.

In experiments by Smellie *et al.* (240) the nucleic acid adenine of malignant tissues (e.g., the Ehrlich ascites tumor) in the intact rat was labeled equally well in the 2- and 8-positions after injection of radioactive formate, but when the tumors were grown *in vitro* formate incorporation into purines occurred only in the 2-position and only to a small extent. Formate incorporation into thymine of DNA proceeded actively in these *in vitro* systems. Formate incorporation into nucleic acid purines (243) was markedly stimulated by aminoimidazolecarboxamide and its riboside, but glycinamide ribotide and riboside had no effect (240). The incorporation of formate into nucleic acid purines *in vivo* was strongly inhibited by azaserine, while the small incorporation observed *in vitro* was insensitive to this inhibitor. This small utilization of formate probably resulted from the conversion to purines of preformed imidazole compounds present in the tissues since the purines formed were labeled only in the 2-position. Their formation from such precursors would not be expected to be inhibited by azaserine. In the presence of liver extracts, which are capable of purine synthesis *de novo*, Ehrlich ascites cells were able to form labeled nucleic acids from C^{14}-formate. Heated liver extracts were partially effective in restoring formate incorporation in the tumor cells also. Lowy *et al.* (244) found that rabbit erythrocytes did not synthesize ATP from exogenous glycine or formate *in vitro*. If 5-amino-4-imidazolecarboxamide riboside were supplied, the adenine portion of ATP was labeled from C^{14}-formate. These results have been interpreted as indicating that most tissues are unable to synthesize purines *de novo* but must rely on external supplies of preformed purines or advanced purine precursors such as the aminoimidazolecarboxamide compounds.

Lajtha & Vane (245) have provided evidence that the liver is the primary tissue for purine synthesis in mammals. They have shown that the nucleic acid purines of rabbit bone marrow of normal animals receiving C^{14}-formate are highly labeled. In hepatectomized animals, however, the incorporation of radioactive formate into nucleic acid purines relative to the uptake of C^{14} into DNA thymine was much lower than in normal animals. This result would seem to indicate that purine synthesis *de novo* normally takes place in the liver and that bone marrow must utilize preformed purines either from this source or from some other store.

Thomson *et al.* (246) and Williams & LePage (247) found that the incorporation of C^{14}-formate and glycine into nucleic acids of ascites cells was decreased when preformed purine compounds were added to the incubation medium. This was probably the result of increasing the size of the acid-soluble nucleotide pool in the cell and of diluting out purines formed *de*

novo. It is also possible, in the light of the work of Wyngaarden *et al.* (64), that the presence of preformed purine compounds suppressed the formation of purines *de novo*.

While the above-mentioned results indicate that *de novo* purine synthesis does not take place extensively in most organs, experiments by Pileri & Ledoux (248) have suggested that cell permeability may be a factor in these incorporation experiments with intact cells. Preformed guanine and adenine were rapidly taken up by HeLa cells growing in liquid culture and were utilized for RNA and DNA synthesis. Formate and glycine, on the other hand, were only poorly incorporated into the acid-soluble pool of the cells and consequently were relatively less important precursors of nucleic acid purines. However, based upon the amount of precursor actually present in the cell, the amount of both glycine and formate incorporated into nucleic acid was comparable to that of the preformed purines so utilized. Degradation studies were not carried out on the products to determine whether both carbon atoms 2 and 8 of the purines were labeled, i.e., whether the purines were formed *de novo*. Experiments bearing on this point have been carried out by Salzman *et al.* (12), who studied the nitrogen precursors of the purines in HeLa cell cultures. Glutamine labeled in the amide nitrogen contributes to two atoms of nucleic acid adenine and three of guanine. It is therefore apparent that *de novo* synthesis of purines can take place in these cells *in vitro*. From this it can be seen that there is some variation in the ability of different experimental tumors to carry out purine synthesis *de novo*.

In all of these experiments one might question the assumption that formate incorporation is a measure of purine synthesis *de novo*. The possibility exists that serine, glucose, or some other metabolite may be the preferred "one-carbon" source in certain tissues, particularly since the introduction of formyl carbon atoms at the 2- and 8-positions of purines involves two different folic acid coenzymes and possibly two different metabolic pathways. Pileri & Ledoux (248) have pointed out that the quantitative interpretation of these results will depend upon a knowledge of pool sizes in the cells. A correlation of the results of the above-mentioned experiments with data from the same tissues in which the permeability barrier has been removed, i.e., in cell-free preparations, would help clear up this question. Also, it is not certain whether comparisons of metabolic activity of tissues grown in culture with that of the same tissue *in vivo* are valid, since many enzyme systems are known to be lost under conditions of *in vitro* culture.

Synthesis of nucleic acids from preformed purines and pyrimidines.— Siegel has measured the incorporation of injected C^{14}-adenine into RNA and DNA of several tissues of young mice (249). In rapidly metabolizing organs such as intestine, liver, and spleen, the ribonucleic acids were labeled more highly than in brain and carcass. DNA synthesis, an indication of mitotic activity, was similarly determined and found to be high in the in-

testine and spleen but relatively low in the carcass, brain, and liver. The specific activity of nucleic acid adenine was approximately the same in RNA and DNA except in liver, where the radioactivity of RNA adenine was several times higher than that of DNA adenine. Williams & LePage have reported studies on the incorporation of purines and their derivatives into the nucleic acids of ascites cells (247, 250, 251). The results indicate that nucleotides must be degraded to nucleosides before they can be taken up by the cells. Potter and co-workers have used regenerating rat liver in studies on nucleic acid synthesis because of its much greater activity in this respect than normal liver. With glucose-1-C^{14} as isotopic precursor, it was found by Schneider & Potter (252) that the rate of synthesis of nuclear and cytoplasmic RNA increases immediately after hepatectomy but that the acceleration of DNA synthesis is delayed for about 18 hr. This delay may represent a period during which new enzyme systems required for DNA synthesis are formed. For example, Canellakis (107) has found that enzymes which phosphorylate thymidylic acid are present in regenerating but not in normal rat liver. Hecht & Potter (253) have shown that the rate of uptake of orotic acid-6-C^{14} into DNA pyrimidines by slices of regenerating rat liver depends upon the rate of DNA synthesis in the liver *in vivo*.

Turnover, metabolic heterogeneity, and conservation of nucleic acids.—Reid & Stevens (254) have fractionated liver RNA into several components and found that they are labeled at differing rates after injection of labeled orotic acid. They suggest that one fraction may be formed directly from acid-soluble precursors and may serve as a precursor of other nucleic acid components. Schneider & Potter (255) have shown that rat liver microsomes labeled with orotic acid-6-C^{14} *in vitro* or in short-term experiments *in vivo* lose much of their radioactivity when they are reincubated in a nonlabeled homogenate. Microsomes isolated from rat liver several hours after injection of the labeled compound do not preferentially lose their label under the same conditions. Evidence was obtained that the "superficially" labeled RNA contains the newly incorporated nucleotides near (but not at) the ends of polynucleotide chains and that at later times these residues may be "buried" deeper in the nucleic acid structure by lengthening of the chain. The results of Breitman & Webster (242) indicate that both the RNA and DNA of isolated calf thymus nuclei are metabolically heterogeneous. Thomson, Paul & Davidson (256) labeled mammalian cells in tissue culture with C^{14}-formate and then reincubated the cells in nonradioactive medium. Both nuclear and cytoplasmic RNA lost part of their radioactive label. DNA did not similarly lose radioactivity unless large amounts of thymidine were present. Under these conditions, DNA thymine was lost but DNA purines were not. This indicates that DNA is turning over but that the products are efficiently reincorporated. Pelc (257) has obtained results which suggest that a turnover of the DNA of mouse seminal vesicle may occur in the absence of cell division. Thus there is evidence that, even

though nucleic acid may be in a metabolically active state, most cells do not lose an appreciable amount of the nucleic acid bases when they are actively growing in an exponential fashion.

In experiments with cultures of Strain L mouse cells labeled with P^{32} and subsequently grown for nine generations in label-free medium, Graham & Siminovitch (258) found a slight initial decrease in RNA-P^{32} and then no further loss in either RNA or DNA radioactivity during the rest of the log-phase multiplication of the cells. Scott & Taft (259) have discussed the general conservation of DNA and RNA in microbial, mammalian, and neoplastic systems. These workers showed that RNA and DNA labeled from orotic acid-6-C^{14} of Ehrlich ascites tumor is conserved even after several generations. Conservation of nucleic acid components seems to be more efficient in tumors and rapidly proliferating cells than in normal animal tissue. De Lamirande et al. (260) found that differences in the activities of purine degradative enzymes may account for these results. In a comparison of several enzymes of normal rat liver with those of the Novikoff hepatoma, it was found that the levels of 5′-nucleotidase, nucleoside phosphorylase, guanase, and adenase were greatly diminished in the tumor and that xanthine oxidase and uricase were completely absent. It was concluded that purine catabolism is completely blocked in the tumor and that purines are probably conserved and recycled. A similar situation may exist in regenerating rat liver with respect to pyrimidine metabolism (107). Bennett & Karlsson (261) injected mice with 2-C^{14}-, 4, 6-C^{14}-, and 8-C^{14}-adenine and found that most of the adenine was retained and not degraded. While this principle of conservation seems to be true with respect to the purine and pyrimidine base portion in many systems, Creaser et al. (262) find that phosphate of nucleic acid is not conserved in ascites cells.

ASPECTS OF NUCLEIC ACID BIOSYNTHESIS IN MICROORGANISMS

Homogeneity and turnover of nucleic acids.—Previous work had indicated that the nucleic acids of exponentially growing bacteria are metabolically stable and do not turn over since there was no loss of radioactivity from either RNA or DNA when the cultures were allowed to grow in non-labeled media (263). These experiments did not rule out the possibility that turnover and efficient reutilization of degradation products were occurring. The metabolic stability, or perhaps more accurately, the conservation (264) of nucleic acids has also been observed in rapidly growing cultures of yeast and mammalian cells (258). However, several cases of RNA turnover have recently been observed under various conditions of metabolic stress, in phage infection (see below) during starvation (265, 266), in unbalanced growth produced by a genetic or metabolic thymine deficiency (267), and also in the RNA produced in the presence of antimetabolites such as chloramphenicol (268) and 8-azaguanine (169). Countryman & Volkin (269) have demonstrated that the RNA of exponentially growing E. coli cultures is metabolically heterogeneous and that interconversion of forms

of RNA may take place. Three fractions of bacterial RNA were obtained by differential centrifugation; P1, which sedimented below 20,000 × g, P2, sedimenting below 140,000 × g, and a supernatant component, S. After a 5 min. incubation of the bacteria in the presence of P^{32}-labeled orthophosphate, the specific activity of P1 was two to three times greater than that of P2 or S. If the cells were exposed to the labeled precursor for only a short time, the specific activity of P1 reached a maximum and then rapidly dropped while the specific activity of P2 and S continued to rise for some time before declining. The activity of DNA was usually equal to that of P2. Fraction P1, which comprised about 5 per cent of the total RNA of the cell, is conceivably a precursor of other nucleic acid components.

Use of chloramphenicol in studying nucleic acid and protein synthesis.— Chloramphenicol has been widely used as a tool in the study of nucleic acid and protein synthesis in bacteria. This compound is considered to be an inhibitor of protein synthesis, although its mechanism of action is not clear. Certain amino acid auxotrophs of *E. coli* will not synthesize nucleic acids in the absence of the required amino acid (270). [Borek & Ryan (271) have described a methionine-requiring mutant of *E. coli* K_{12} which is an exception to this rule.] When catalytic amounts of the required amino acid or of certain amino acid analogues were supplied, RNA synthesis was restored (270). According to Gros & Gros (270), these results suggest that free amino acids are involved in some way in RNA synthesis and that concomitant protein synthesis is not required. Furthermore, when chloramphenicol was added in the presence of the required amino acid (or to wild-type *E. coli*) net RNA synthesis could take place in the absence of detectable incorporation of amino acids into protein. The RNA formed in the presence of chloramphenicol is unique in that it is largely (50 to 60 per cent) degraded when the chloramphenicol is removed (268, 272). This RNA also differs from normal RNA in electrophoretic mobility, ultracentrifugal sedimentation rate, and ease of dissociation of its nucleoprotein complex (273), although its base composition is the same as that of normal RNA (272). Aronson & Spiegelman (274) have critically re-examined the results and conclusions derived from previous experiments with chloramphenicol since they had found that the low levels of inhibitor normally used did not prevent protein synthesis completely. At high concentrations of chloramphenicol, the amino acid requirement for RNA synthesis in amino acid auxotrophs could be abolished. From their results Aronson & Spiegelman concluded that the "unstable" RNA formed in the presence of chloramphenicol is a normal stage in the formation of a stable ribonucleoprotein since (*a*) the formation of an unstable RNA could be detected in short-term experiments in the absence of chloramphenicol, and (*b*) "unstable" RNA formed in the presence of the inhibitor could be partially converted to a stable form if protein synthesis were allowed to take place by supplying amino acids after the chloramphenicol was removed. Under these conditions amino acids were incorporated into particle-bound proteins without any incorporation of

precursors into the nucleic acids. While this explanation of the action of chloramphenicol on RNA synthesis in bacteria seems reasonable, it is not yet understood how chloramphenicol replaces the amino acid requirements for RNA synthesis or how the blockage of protein synthesis occurs.

Interdependence of nucleic acid and protein synthesis.—The mode of action of chloramphenicol may be somewhat different in other organisms than in the strains of *E. coli* and *Bacillus megatherium* which have been studied. Protein and DNA synthesis in *Azotobacter agilis* were blocked in the usual manner by low concentrations of the inhibitor, but RNA formation was also decreased at higher concentrations (275). Cobalt and uranyl ions inhibited RNA, DNA, and protein synthesis more or less equally in *A. agilis*. Breitman & Webster have found that chloramphenicol inhibits protein, DNA, and RNA formation equally in pea seedling and mammalian systems but that much higher concentrations of inhibitor were required than in the bacterial system (276, 277). A possible interrelationship, in some systems, between nucleic acid and protein synthesis is suggested by these results.

The RNA formed in *E. coli* in the absence of protein synthesis does not support growth and multiplication when the chloramphenicol is removed. Hahn *et al.* (278) concluded that most of this RNA is lost (i.e., degraded) before growth resumes and that the fragments are then reincorporated into nucleic acids. In contrast to the RNA formed in the presence of chloramphenicol, that formed by the methionine deficient *E. coli* mutant, investigated by Borek & Ryan, was stable after the cells were replaced on the complete medium (271). The accumulated RNA appeared to be nonfunctional, although its composition was the same as that of normal RNA. If methionine were supplied to bacteria which had been allowed to accumulate RNA, there was a long lag period during which more RNA was formed. Ben-Ishai (279) has reported that when protein synthesis was resumed after methionine starvation or aureomycin inhibition in *E. coli*, the rate of formation depended not on the total RNA present but on the rate of RNA synthesis. In these experiments the cells were allowed to form RNA in the presence of an inducer of adaptive enzyme formation but under conditions in which protein synthesis was prevented. If the inducer were then removed and the conditions for protein synthesis were restored, there was no formation of the specific adaptive enzyme. These results emphasize the necessity for the inducer to be present at the time of enzyme synthesis. Barner & Cohen (267) have studied the relationships between protein and nucleic acid metabolism in a double mutant of *E. coli* 15 which requires uracil and thymine. The omission of thymine from the medium containing cell suspensions prevented DNA but not RNA or protein formation and the absence of uracil completely inhibited net RNA synthesis. Net protein and adaptive enzyme formation could occur when both thymine and uracil were omitted and when total nucleic acid formation was inhibited. Although protein synthesis could take place in the absence of net nucleic acid formation, it could be

shown that a portion of the RNA was being degraded and resynthesized. Presumably this turnover of RNA could be involved in the formation of new protein. It would be of interest to know whether the observed RNA turnover would take place in the absence of protein synthesis, as in a mutant with a superimposed genetic amino acid deficiency. Okazaki & Okazaki (280) have found that a somewhat different situation exists in *L. acidophilus,* an organism with pyrimidine deficiencies similar to those of the double mutant studied by Barner and Cohen but which has additional requirements for deoxyribosides and certain amino acids. DNA synthesis and cell proliferation were almost completely abolished in the absence of thymine, but RNA and protein formations were only slightly decreased. If either uracil or a required amino acid were omitted, both RNA and protein formation ceased but DNA continued to increase.

The results summarized above may be interpreted to mean that in these bacteria the synthesis of both constitutive proteins and adaptive enzymes are dependent upon the synthesis of RNA and not upon the RNA content of the cells. In particular, Okazaki and Okazaki's report suggests a close interrelationship between RNA and protein synthesis. On the other hand, Nomura & Hosoda (281) have concluded from studies on amylase formation in *B. subtilis* that the formation of this enzyme requires the presence of RNA but does not necessarily depend upon RNA turnover or its continued synthesis.

E. coli cells treated either with ultraviolet light or nitrogen or sulfur mustards behave similarly in several respects, according to studies by Harold & Ziporin (282, 283, 284). DNA synthesis is transitorily inhibited, but RNA and protein formation are not. This unbalanced condition of growth may result in abnormal morphology and loss of viability if the dosage of the mutagenic agent is large. Harold & Ziporin found that a period of protein synthesis preceded the formation of new DNA in cells recovering from the effect of these agents. Recovery of the ability to form DNA was prevented if protein synthesis were blocked in amino acid-requiring mutants or by amino acid analogues or by chloramphenicol. If chloramphenicol were added at various times during the recovery period so that protein synthesis was blocked at different stages of completion, the subsequent rate of DNA formation depended on the amount of new protein which had been formed (284). It would appear that these agents specifically destroy, or irreversibly inhibit, enzymes necessary for DNA synthesis. The fact that the mutability of bacteria induced by ultraviolet irradiation depends on the rate of protein synthesis immediately after irradiation further points out the role of protein in DNA synthesis. These results may be related to the fact that x-ray irradiation of rats inhibits the ability of bone marrow cells to form DNA. The possible site of inhibition was suggested by the observation that a supply of deoxyribosides was able to restore DNA formation in x-ray damaged cells (285).

Nucleic acid synthesis in virus infection.—A number of reports have ap-

peared dealing with the sequence of events in protein and nucleic acid synthesis accompanying bacteriophage infection. The most thoroughly investigated system is that of T2 infection of *E. coli*. Immediately after infection net change in the RNA content of the culture ceases, but a rapid turnover of RNA begins (286 to 288). The new RNA formed in this process is different from the host RNA and is characteristic of the phage (289, 290). Associated with this process is an increase in protein. Part of this protein is not incorporated into the new phage particles but is necessary for the synthesis of phage DNA. If chloramphenicol is present during the early stages of infection, RNA turnover, the net synthesis of protein and, as a result, the formation of phage DNA are prevented (286, 287, 290, 291). If chloramphenicol is added after protein synthesis has started, DNA synthesis can take place at a rate dependent upon the amount of new protein formed. After this initial phase of RNA turnover and protein synthesis, these processes slow down while the period of rapid DNA synthesis commences. Much of the nucleotide material incorporated into RNA (measured by P^{32} or C^{14} labeling) during the initial RNA turnover is transferred to the new DNA being formed (287). Nucleotides may be directly incorporated into the DNA without first being converted to RNA, however (288). Thus, Watanabe *et al.* (290) have found that if chloramphenicol were added after the phase of protein synthesis the breakdown of RNA and transfer of P^{32} from RNA to DNA was inhibited. Under these conditions, DNA synthesis involved nucleotide sources other than RNA. Jeener (292) has observed that the formation of specific phage proteins is prevented after induction of a lysogenic *B. megatherium* in the presence of thiouracil or azaguanine. These agents either block the synthesis of RNA or result in the formation of altered RNA.

In certain phages DNA formation appears not to require prior protein synthesis, since DNA can be formed even if protein synthesis is blocked completely by chloramphenicol. Crawford (293) reported earlier that T1, T3, T5, T7, 201S, and C1 coliphages were of this type, but that the T-even phages all were similar to T2. It was originally suggested that the preliminary protein synthesis involved in infections by T-even phages, which contain the pyrimidine base 5-hydroxymethylcytosine, might be confined to the formation of enzymes required for the synthesis of this compound. It has now been found, however, that both T5 (294, 295) and the M4 (295) virus of *B. megatherium,* neither of which contain hydroxymethylcytosine, also require protein synthesis prior to DNA formation. It would seem likely that a more general alteration of protein synthesis is necessary in these cases and that certain phages may be able to use the enzymatic machinery of the host cells to make their own DNA without the formation of appreciable amounts of new enzymes. Infection of *E. coli* by T5 results initially in the rapid breakdown of host DNA as well as RNA followed by the reincorporation of the fragments into phage DNA (294, 296).

Some studies have been made of nucleic acid metabolism during the

multiplication of plant and animal viruses, but our understanding of these processes is still in a comparatively primitive stage. During the early stages of poliomyelitis virus infection in HeLa cells both RNA and DNA syntheses are inhibited, but at a later stage the rate of RNA synthesis increases while that of DNA continues to decrease (297). The preferential synthesis of RNA in this RNA-containing virus is analogous to the enhanced synthesis of DNA in bacteriophage [Goldfine *et al.* (297)]. Staehelin has studied the incorporation of P^{32} into tobacco mosaic virus RNA (298, 299). Upon degradation of the RNA to 5′-nucleotides a wide variability in labeling of the various products is found, while on degradation to the 2′- and 3′-phosphates, the nucleotides are found to be uniformly labeled.

Occurrence of New Bases in RNA and DNA

Recently improved methods of isolation have permitted the identification of several new components in nucleic acids. The occurrence of an abnormal uridylic acid in RNA had been reported by Davis & Allen (300) and by Cohn (301). This compound may constitute up to 9 per cent of the nucleotide content of some fractions of yeast RNA. Cohn has isolated the material as a nucleoside from RNA by treatment of RNA with hydrogen fluoride and believes it to be 5-ribosyluracil (308). This compound has been derived from pancreatic RNA by Kemp & Allen (302), who also have reported the isolation of three additional compounds with guanine-like spectra. Cohn also finds two or three methylated adenine derivatives in hydrogen fluoride digests of RNA. These compounds may be identical with the methylated purines isolated from nucleic acids by Adler *et al.* (303), by Dunn & Smith (304), and by Littlefield & Dunn (305, 306). The former workers have found trace amounts of 6-methylaminopurine, 6-hydroxy-2-methylaminopurine, and 1-methylguanine in yeast RNA but not in DNA. Littlefield and Dunn have detected 6-methylaminopurine, 6,6-dimethylaminopurine, and 2-methyladenine in RNA of bacterial origin in amounts of 1/10 to 1 per cent of the uracil present. The former two compounds are also present in rat liver RNA. 6-methylaminopurine is present in the DNA of *E. coli* to the extent of 2 per cent of the adenine (304). Littlefield and Dunn report, in addition to the three methylated purines, the presence of thymine in the RNA of yeast, *E. coli*, and *A. aerogenes*. Amos & Korn (307), on the other hand, find that 5-methylcytosine constitutes 1 to 2 per cent of the RNA nucleotides from *E. coli* and that during acid or alkaline hydrolysis this compound is deaminated to thymine. These workers suggest that the thymine constituent in RNA reported by Littlefield and Dunn may actually be a degradation product of 5-methylcytosine.

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WATER-SOLUBLE VITAMINS, PART I[1,2,3]

THIAMINE, RIBOFLAVIN, PANTOTHENIC ACID, NICOTINAMIDE, LIPOIC ACID (THIOCTIC ACID)

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As it was possible to discuss only a portion of the papers published in this field during the past year, it is obvious that some worthwhile investigations have not been mentioned. May we be forgiven the unavoidable inequities resulting from our choice. The major emphasis has been on papers that are related to biological applications.

THIAMINE

Chemistry and methods.—The nonenzymatic reaction of thiamine with pyruvate has been reported by Yount & Metzler (1) to produce α-acetolactate and acetoin. In borate buffer, the acetolactate formed is rapidly decarboxylated to acetoin.

Bonvicino & Hennessy (2) have shown that thiamine is reduced by sodium borohydride to a tetrahydro and a dihydro derivative, and by sodium trimethyoxyborohydride to a lower-melting dihydrothiamine. Lhoest *et al.* (3) studied the degradation of thiamine as a function of pH by paper chromatographic methods to show a progressive formation of the carbinol form, thiochrome, probably thiamine disulfide, two pyrimidine products, and two unknown products. They also reported on the sulfite cleavage products of thiamine (4).

Breslow (5) has continued his work with model systems. The fact that N-benzyl-(α-d2)-thiazolium bromide used as a catalyst does not lose deuterium shows that catalysis does not involve condensation with the N-

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methylene group. Other considerations, including the finding that while thiamine pyrophosphate has catalytic properties, thiamine disulfide has not, and the fact that anions on doubly bonded carbons can be relatively stable, the stability being enhanced by a positive nitrogen, suggested that a zwitterion (I) might be formed by loss of a proton from a thiazolium salt under relatively mild conditions.

I
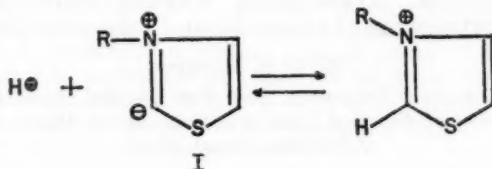
The existence of such a zwitterion was detected by deuterium exchange. That the hydrogen at C-2 is exchanging was shown by infrared and nuclear magnetic resonance evidence.

The fact that thiazolium salts are in equilibrium with anions at C-2 under mild conditions seems to account for the catalyses which have been observed. The structural similarity of zwitterion (I) and cyanide ion is noted, and it is suggested that catalysis by thiazolium salts occurs via zwitterion (I) in a fashion analogous to catalysis by cyanide ion. The results with simple chemical model systems appear likely to reflect the processes involved in biochemical reactions, but only further work will show whether or not they have furnished a true insight into the mechanism of thiamine action.

Thiamine triphosphate-P^{32} has been prepared (6) from the diphosphate and phosphoric acid by using N-N¹-dicyclohexylcarbodiimide. The activity of the γ-P was determined after reaction with myosin (7) which can hydrolyze thiamine triphosphoric acid at pH 9.1, and the β-P was determined by hydrolyzing with 0.3 N HCl. Relative values obtained for α-, β-, and γ-P were 0, 7040, and 13,680 impulses per minute, respectively.

The oxidation of ascorbic acid to dihydroascorbic acid can be prevented by thiamine and by oxythiamine, an antioxidant property associated with the thiazole part of the molecule (8). This effect will not be hindered by Cu ions. Experiments on guinea pigs showed that thiamine administration increased the content of ascorbic acid in the organs and muscles of both normal pigs and animals on a scorbutic diet.

The reducibility of allithiamine by sulfhydryl compounds to form allyl disulfide and thiaminyl thiol has been used by Kato (9) as the basis of a method for the microdetermination of sulfhydryl groups in metamorphosing insects.

Thiamine and TPP in tissue extracts can be separated by passing the deproteinized extracts (pH 5.9) through an Amberlite IRC-50 column on which thiamine is strongly adsorbed (10). TPP can be eluted with distilled water. Dilute hydrochloric acid will remove the thiamine in the fraction be-

tween pH 4.0 and 2.1. A technique for purifying crude TPP from large amounts of thiamine, di-, tetra-, and pentaphosphates by column chromatography has been described by Suzuoki *et al.* (11).

Recent reports of analytical methods for thiamine and its derivatives include polarimetric estimations (12) and paper ionophoretic separations of thiamine from thiamine propyl disulfide (13, 14). Pleticha combined paper-chromatographic and polarographic techniques to claim superior precision in the analyses of old contaminated thiamine samples (15) and of yeast (16). Segre *et al.* (17) described a paper electrophoretic separation of the mono-, di-, and triphosphoric esters of thiamine; Yoshida (18) and Wakisaka & Ishida (19) used ion exchange chromatography to attain the same end.

Among enzymatic techniques recently recorded, Horie (20) has modified the manometric method for the estimation of TPP, and Kaziro (21) has devised a new photometric technique for its determination which places variations in the reaction rate of apocarboxylase on a quantitative basis.

Developments in microbiological estimations include the report of Deibel *et al.* (22) using *Lactobacillus viridescens* in which TPP has about 60 per cent of the activity of thiamine. The pyrimidine and thiazole moieties are not utilized in this assay. Improvements in the *Lactobacillus fermenti* assay have been claimed by Maciasr (23) and also by Edwards *et al.* (24). The latter applied it to analyses of blood samples. Jansen *et al.* (25) have a microbiological assay for 0.2 ml. of blood which uses a mutant of *Escherichia coli* as the test organism. Bechtel & Hollenbeck (26) have proposed a simplified thiochrome procedure which should prove useful in the routine analyses of cereal products.

Westenbrink (27) has reviewed the biosynthesis, breakdown, phosphorylation, and cellular distribution of thiamine. Mickelsen & Yamamoto (28) have published a comprehensive review of analytical methods. This gives a useful description of animal, microbiological, enzymatic, and chemical techniques developed through the years and includes many useful hints for the analyst.

Thiaminase and thiamine antagonists.—Japanese workers have continued the interesting studies reviewed by Hayashi (29) on the destruction of thiamine by bacterial enzymes. Fujita (30) has discussed the two types of thiaminase, one which catalyzed the base exchange reaction of thiamine, and a second which catalyzed the hydrolysis of thiamine to its pyrimidine and thiazole moieties. Murata & Ikehata (31) noted that the decomposition of TPP did not occur with thiaminase II but that bacterial thiaminase I readily changed TPP to a pyrimidine derivative and thiazole pyrophosphate. Murata (32) reported that thiaminase II of *Bacillus aneurinolyticus* also failed to decompose TPP, whereas thiaminase I of *Bacillus thiaminolyticus* and of shell fish directly catalyzed a base exchange reaction. The majority of yeast-like fungi have thiaminase activity [Ozawa *et al.* (33)]. As 2-ethylthiothiamine has been found to be a reversible antagonist of thiamine in microorganisms, Sakuragi (34) compared the antithiamine activities of the 2-

alkylthio analogues of thiamine on *Kloeckera brevis* and *L. fermenti* 36 with pyrithiamine and oxythiamine. For *K. brevis,* 2-methylthiothiamine was more effective than pyrithiamine, which, in turn, was more effective than the 2-ethylthio-, oxy-, and 2-*n*-propyl- analogues; for *L. fermenti* 36 the order of decreasing effectiveness was: pyrithiamine > 2-methylthiothiamine > 2-ethylthiothiamine > oxythiamine > 2-*n*-propylthiothiamine. Siva Sankar (35) showed that the oxythiamine toxicity in *Neurospora crassa* could be completely reversed by acetate as well as by thiamine. Succinate and citrate could also reverse this inhibition, which may be ascribed to the formation of acetate from these compounds. The two thiaminases in the fresh water mussel, *Lamellidens marginalis,* were separated [Giri (36)] after adsorption on alumina. One has optimum stability at pH 6.5 and can be eluted with phosphate buffer at this pH; the other ,which has optimum stability at pH 3.6, is not eluted. The thiaminase content of a number of fresh and salt water fish was studied by Deolalkar & Sohonie (37). Fish from fresh water appeared to contain only one thiaminase with optimum activity at pH 7.0, whereas salt and brackish water species had enzymes active at pH 3.6 and 5.6. Manganese and cobalt were needed to activate the latter but not the thiaminase at pH 7.0. Some aromatic amines, i.e., aniline, toluidines, and pyridine, were capable of reactivating these enzymes after they were boiled (38). Nakabayashi (39) isolated from horsetails (spore stalks of *Equisetum arvense,* used in Japan as a vegetable) two thermostable substances which decompose thiamine. One was pentahydroxyanthraquinone and the other its glucoside. Hydroxyanthraquinones occur widely in animals, plants, and microorganisms. Another thermostable compound was isolated from sweet potato leaves by Sakamoto & Fujita (40). This also has thiamine-decomposing properties and was identified as the flavonol isoquercitrin. The list of thermostable antithiamines now includes flavonoids, phenols, and tannins.

Reports on the thiamine inhibitory activities of pyrithiamine and oxythiamine in mammalian tissues continue to show separate metabolic mechanisms for these two compounds. Studies of the respiratory quotient and heat production after glucose administration to rats by De Caro *et al.* (41) confirmed the consideration that the normal responses to glucose were inhibited by pyrithiamine but not by oxythiamine. They also studied (42) the effects on dietary avitaminosis to show that neopyrithiamine action expresses itself mainly as an impoverishment of the tissues in phosphorylated thiamine. An increase in the glutamic-pyruvic transaminase activity of rat liver was noted by Perri & Rindi (43) after neopyrithiamine administration, but the glutamic- oxaloacetic transaminase activity was not affected. Gubler (44) reported on the varying effects of thiamine deprivations and oxythiamine or pyrithiamine administration on α-ketonic acid oxidation in rat tissues to show marked differences. For example, in brain homogenates, pyrithiamine administration reduced the oxidative rates for pyruvate and α-ketoglutarate to about 50 per cent each, but no such changes were noted after thiamine deprivation and oxythiamine. Similar studies on rat erythrocytes by Wolfe

(45) showed that when rats were given oxythiamine, their red cells exhibited defective methylene blue-activated glucose oxidation like those from thiamine-deficient animals. This effect could not be reversed by incubation of oxythiamine-affected cells with large amounts of thiamine or TPP. Erythrocytes from pyrithiamine-treated rats, although they developed a severe neuritis, did not show any altered glucose metabolism. In pigeons [Paroli (46)], oral administration of neopyrithiamine produced typical symptoms of avian polyneuritis, but the symptoms produced by intramuscular injection were not characteristic of beri-beri.

Thiamine synthesis by intestinal microflora.—The possible contribution of intestinally synthesized thiamine to the nutrition of the host remains an important practical problem. Mameesh & Johnson (47) used C^{14}-labeled thiamine to demonstrate that in rats receiving a thiamine-limiting diet the available microbial thiamine was approximately 4 and 1 μg. per day, with and without penicillin in the diet, respectively, while in rats which received an adequate diet the values were 6 and 0 μg., respectively. The diet used contained 73 per cent sucrose. Similarly, the treatment of sheep with chlortetracycline (aureomycin) (30 mg. per kg. for 9 days) raised the thiamine content of the animal rumen about 11 per cent.

Balakrishnan *et al.* (48, 49) compared thiamine biosynthesis in rats on diets with and without thiamine, milk curds, and sulfaguanidine, respectively. Intestinal thiamine synthesis appeared to be increased by the curds alone, but sulfaguanidine caused a significant decrease accompanied by a diminished growth rate. Sulfaguanidine given together with milk curds showed a decreased inhibition of thiamine synthesis and produced a normal growth rate. Thiamine administration was claimed to decrease the intestinal synthesis of thiamine.

The marked effectiveness of penicillin in overcoming stresses that normally increase the thiamine requirement was demonstrated by Vogel *et al.* (50). They fed thyroprotein (iodinated casein) to rats on varied thiamine intakes to obtain significant decreases in the growth rate. The addition of penicillin G to the diet of these animals markedly increased the growth rate and decreased the elevated oxygen consumption caused by the thyroprotein.

The sparing effects of ascorbic acid on the effects of thiamine deficiency in the rat has been studied further by Terroine (51). He also reports that ascorbic acid stimulated the growth of *L. fermenti* 36 in a thiamine-deficient medium. Morgan & Yudkin (52) investigated the sparing action of sorbitol in rats to show that when the glucose component of a thiamine-deficient diet, on which the animals died in 5 to 10 wk., was replaced by sorbitol, the animals gained weight and survived for 30 wk.; and although the caecum was greatly enlarged, there was no more thiamine in the tissues than in rats which received glucose. A comparison of the effect of banana and rice in the rat (53) showed some improvement of growth with the banana diet.

Wacker & Pfahl (54) reported on the biosynthesis of thiamine deoxyriboside by *E. coli* 113-4 using C^{14}-labeled uridine and uracil.

Thiamine metabolism.—The possible role of thiamine in the oxidation of tryptophan to tryptophan peroxidase-oxidase was carefully investigated by Townsend & Sourkes (55), and the results do not support the hypothesis that thiamine is involved in this reaction.

Iacono & Johnson (56) studied the metabolism of thiazole-2-C^{14}-thiamine in the rat to show that 60 per cent of the radioactivity of intraperitoneally injected thiamine was excreted in the urine. At least 15 other radioactive metabolites were separated by paper chromatography; among these thiamine disulfide, thiochrome, and the thiazole moiety of thiamine were identified. Several others stimulated *L. fermenti* 36 on a thiamine-deficient medium. Verrett & Cerecedo (57) reported on the fate of S^{35}-labeled thiazole in rabbits and obtained 77 per cent of the radiosulfur in the urine and feces after administration by stomach tube, 86 per cent after intramuscular administration, and 54 per cent after intravenous injection. The oral administration of a twentyfold larger dose of nonradioactive thiamine caused an additional excretion of 17 per cent of the radiosulfur. The unchanged thiamine-S^{35} and thiazole-S^{35} accounted for about 90 per cent of the orally administered radiosulfur. That the sulfur of DL-methionine-S^{35} can be incorporated into thiamine was shown by Yamada *et al.* (58).

Brin *et al.* (59) studied the effect of thiamine on the glucose oxidative pathway in erythrocytes and reported that rat erythrocyte transketolase activity in the presence of methylene blue is reduced on a thiamine-deficient diet. In severely deficient erythrocytes, pentose accumulated to three times the normal level, and the recovery of $C^{14}O_2$ from glucose-2-C^{14} was depressed to one-seventh of normal.

Kiessling (60, 61) investigated the incorporation of P^{32} from inorganic phosphate into TPP in rat and guinea pig liver mitochondria. Most of P^{32} was incorporated into the β-P. The effect of substrates, such as fumarate and succinate, was interpreted to indicate that the mechanism does not depend on the transformation of succinate into pyruvate, although previous work (62) had shown that the incorporation of P^{32} into TPP appeared to be dependent upon the presence of pyruvate as a substrate.

Thiamine nutrition and physiology in man.—The latest edition of *Recommended Dietary Allowances of the National Research Council* (63) retains the recommendations for thiamine as made in 1953. Young and his associates (64, 65) studied the food intake of new college students by making dietary records of food consumption for seven days and concluded from the sample used that about 22 per cent of the girls had thiamine intakes below 80 per cent of the Recommended Dietary Allowances and about 18 per cent of the young men studied were consuming less than 70 per cent of the recommendations. Hart & Reynolds (66) interpreted their studies of urinary excretion on nine girls between the ages of 16 and 18 as showing that an intake of 0.3 mg. per 1000 cal. was inadequate and that 0.6 mg. per 1000 cal. was marginal.

A conference on beri-beri in terms of modern nutritional, clinical, and biochemical problems was held at Princeton, New Jersey, in June, 1958.

The proceedings (67) of this symposium are recommended for discussion of the many facets of this disease. Baron & Oliver (68) reported on a case of fulminating beri-beri in a British resident. It is interesting how few such cases are available for teaching purposes in the modern occidental world, a testimonial to the success of supplementation procedures.

Brozek (69) has reported on the psychological effects of thiamine restriction on young men to show that tests of intelligence were not affected adversely by thiamine deprivation but that consistent changes were obtained in pressure- pain thresholds, motor reaction times, eye-hand co-ordination, and manual steadiness.

The oft repeated claim that thiamine in amounts greater than the "daily requirement" improved the performance of athletes was made again in a report by Vytcikova (70), who judged thiamine needs by studying pyruvic acid and thiamine levels in the blood and urine. He suggested that 1.5 to 2 mg. was inadequate on a diet of 3700 to 4000 cal. and claimed that this should be increased to more than 10 mg. per day for distance runners and basketball players.

Trying to evaluate the incidence of dental caries as a function of the dextrinizing time of salivary amylase with a corn starch substrate, Turner et al. (71) pointed to the pronounced increase in the rate of dextrinization when thiamine was added. They noted that thiamine speeded up the rate of dextrinization more for the caries-resistant children than for caries-prone patients.

Ruggieri & Smilari (72) studied the effects of intravenous administration of 50 mg. of TPP and noted an excitatory effect on gastric hydrochloric acid excretion in some normo- and hypo- but not in hyperchlorhydric subjects.

Investigations of the influence of food preparation on thiamine loss include the studies of Coppock et al. (73), who showed that during bread baking approximately 15 per cent of the thiamine was lost; Pai et al. (74) reported on the thiamine content and cooking losses of a number of Indian foods, showing that cooking losses ranged from 2.6 to 57 per cent and, as might be expected, the extent of loss depended upon washing procedures and the temperature and duration of cooking.

Thiamine nutrition and physiology in animals.—The effect of low environmental temperatures on the weight and food consumption of thiamine-deficient rats was studied by Vaughan & Vaughan (75), and no apparent special influence of thiamine was noted. Sekun (76) studied the gastric secretions of thiamine-deficient dogs to find that the digestive activity of secretions from gastric pouches of these dogs had a greater than normal volume in response to a meat meal but that the digestive activity of such secretions was decreased.

The efficiency of absorption as a function of age was investigated by Draper (77) in order to test the hypothesis that older rats have a larger thiamine requirement than younger animals. Using an oral dose of 120 µg.

of C^{14}-labeled thiamine, he showed that 95 per cent was absorbed up to age 19 to 20 months and that this declined to 75 per cent at 22 to 24 months. Thiamine deficiency was shown by Ferrari (78) to cause a pronounced decrease of DPN in rat liver.

Studies of the effects of thiamine deficiency on cardiovascular function included that on rats by Beznak (79) in which no differences in acetylcholine content of hearts of deficient and pair-fed controls were noted, both showing a definite bradycardia; after vagotomy (80) which quickens the heart rate in normal rats, deficient rats showed a further decrease of bradycardiac beats. The marked blood vessel constriction in rabbit ear veins caused by perfusing with nicotine is diminished by thiamine (81). The electrocardiograms of deficient piglets were described by Miller *et al.* (82) to show considerable heart damage after 5 weeks on a diet low in thiamine.

Among studies of brain and nerve function related to thiamine metabolism is the report of North & Sinclair (83) on degenerative changes in the peripheral nerves. These changes do not become apparent except in severe, prolonged deficiency in the rat. The forms of thiamine in rat sciatic nerves were studied by electrophoresis of nerve extracts by Gertner (84). Strong electrical stimulation for 1 min. markedly increased the free thiamine and TP content and decreased the amount of TPP and TPPP. Doi (85) reported that the injection of 2 mg. per kg. of thiamine or thiamine propyldisulfide to rabbits did not raise the thiamine content of the blood even after 60 days but that the amounts in the neural tissues were increased with successive administrations. The latter effects should not be surprising, since blood thiamine concentrations are notoriously poor as indicators of thiamine saturation.

Chickens and pigeons, classical experimental material in studies of thiamine nutrition, continue to provide interesting information. Howes & Hutt (86) studied genetic variations in the efficiency of thiamine utilization of 13 strains of White Leghorn chickens and of 13 strains representing four heavy breeds to confirm the fact that White Leghorns put more than 40 per cent more thiamine into their eggs. It was concluded that these birds have a lower requirement of thiamine both as chicks and as adults. Nobile *et al.* (87) noted that in pigeons on a polished rice diet, those that were supplemented with 50 mg. of choline chloride per day developed thiamine deficiency faster than birds unsupplemented with choline.

RIBOFLAVIN

Chemistry and methods.—If an acqueous solution containing 10^{-3} M tryptophan and 10^{-3} M riboflavin-5^1-phosphate is frozen, the resultant sample is red instead of yellow. The red form of this mixture can also be formed at room temperatures if 10 times more concentrated solutions of tryptophan are used. Isenberg & Szent-Györgyi (88) have presented evidence that this phenomenon, which is also shown by protein, serotonin, lysergic acid, bufotenine, and other indole derivatives, is caused by the change of riboflavin,

after taking up an electron from tryptophan to a semiquinoid form, a free radical. They state that such complex formations might be much stronger in tissue than *in vitro*. If electrons lost from protein are replaced by electrons given off by dehydrogenated metabolites, DPN or TPN, then the electron transport might be interpreted as going through the protein molecule itself and not merely by action on the surface of the molecule, as hitherto believed.

The photolysis of riboflavin was studied by Sakai (89) to show that aerobic, but not anaerobic, photolysis produced formic acid and traces of glycolic acid as a result of the production of hydrogen peroxide; this change was accelerated by iron compounds. Rajewsky *et al.* (90) investigated the effects of x-ray irradiation by which riboflavin was converted to a compound that had no absorption between 230 and 500 mμ. If 3 per cent albumin was added to the riboflavin solutions, four times as much radiation was needed to give the same effect. Kameda (91) reported that the addition of ferrous sulfate (10^{-4} M) to solutions of riboflavin compounds shifted their maximum absorption bands from 450 to 430 mμ and decreased fluorescence. The maximum absorption of FAD was shifted to 460 mμ by the addition of D-amino oxidase.

A method for determining phosphorylated riboflavin in the presence of vitamin B_{12} and hematoporphyrin was described by Ruggieri (92). After oxidation with permanganate and removal of excess permanganate with hydrogen peroxide, optical densities were read at 350 to 360 mμ. Simplified procedures for liberating riboflavin from natural feedstuffs to facilitate routine analyses were described by Murthy *et al.* (93). For fluorometric determinations, samples were suspended in 0.06 per cent hydrochloric acid, steamed in an autoclave for 15 min., cooled quickly, then diluted and centrifuged. For microbiological assay the procedure was similar, except that water instead of dilute hydrochloric acid was employed.

Riboflavin metabolism of microbiological material.—*Eremothecium ashbyii* continues to provide reliable data on riboflavin biosynthesis. Using this yeast, Brown *et al.* (94) report that purines fall into the following order of decreasing effectiveness as stimulators of riboflavin synthesis: guanine, xanthine, adenine, hypoxanthine, and uric acid. Adenine is converted partly into hypoxanthine, partly into riboflavin, and some remains unchanged. McNutt & Forrest (95) also investigated how *E. ashbyii* incorporated the pyrimidine ring of adenine into the pyrimidine portion of the isoalloxazine ring of riboflavin. They found a compound which, it was hoped, might be an intermediate between adenine and riboflavin and which has a purple fluorescence in ultraviolet light; but they were not able to prove that this compound, a new crystalline pteridine (96), was a precursor of riboflavin.

Maley & Plaut (97) using *Ashbya gossypii,* found a green-fluorescent compound with the structure, 6,7-dimethyl-8-ribityl-lumazine. This compound cannot replace riboflavin in the *Lactobacillus casei* assay. Smyrniotis *et al.* (98) used a pure unknown strain of a riboflavin decomposing bacter-

ium to produce 3,4-dimethyl-6-carboxy-α-pyrone. They believe that this py-
rone is derived from riboflavin with the intermediary formation of dimethyl-
pyrogallol.

The chemical properties of riboflavinyl glucoside synthesized from mal-
tose and riboflavin by *E. coli* were reported by Katagiri *et al.* (99) to be
identical to those derived by rat liver enzymes or by *Aspergillus oryzae.*

Snoswell (100) demonstrated the presence in cell-free extracts of cul-
tures of *L. arabinosis* 17-5 of FMN and FAD with little or no free ribo-
flavin. The flavokinase responsible for the synthesis of these nucleotides was
isolated and found to require a high-energy phosphate donor and a divalent
metal ion, such as ATP and Mg^{++}, for optimum activity.

Repeated exposure of *L. casei* to increasing levels of the riboflavin in-
hibitor, 6-chloro-7-methyl-9-(1′-D-ribityl)-isoalloxazine, was reported by
Scala & Lambooy (101) to modify this bacterium so that it accepted either
riboflavin or its analogue as the sole source of riboflavin.

Thimann & Radner (102), while studying the biogenesis of anthocyanin
in plant materials, noted that the formation of anthocyanin is quantitatively
controlled by the riboflavin content of the plant. When plants were pre-
illuminated, riboflavin increased anthocyanin production in a subsequent
dark period. It was concluded that riboflavin does not act as a photoreceptor
but as a dark catalyst to produce anthocyanin from sucrose or other pre-
cursors, each molecule of riboflavin leading to formation of 30 to 60 mole-
cules of anthocyanin.

Lascelles (103) has shown that the synthesis of porphyrins from δ-amino
laevulinic acid cell suspensions of *Tetrahymena vorax* is decreased in the
absence of riboflavin but is not affected by deficiencies of pyridoxal, panto-
thenic acid, nicotinic acid, thiamine, or lipoic acid.

Stadtman (104) has summarized significant developments in recent re-
search on the biosynthesis and degradation of riboflavin. This review in-
cludes a detailed description of the role of purines in ring formation,
making use of the synthetic capabilities of *E. ashybii* to study the similari-
ties in the structure of purines, pyrimidines, and riboflavin which all con-
tain the diazine ring.

Riboflavin in human nutrition and metabolism.—Tucker *et al.* (105)
have reported on the effects of sleep, work, heat, and diuresis on the ribo-
flavin excretion in men. When the men were exposed to high temperatures
or hard work, they observed an increased excretion. This might be directly
correlated with the simultaneous changes in nitrogen excretion, although
this was not specifically studied. The data confirm the absence of correla-
tion (106) between the volume of urine and the amount of riboflavin ex-
creted.

An excellent analysis of the literature on riboflavin requirements of man
and animals was prepared by Bro-Rasmussen (107, 108) in which the inter-
dependence of riboflavin and protein utilization received prominent at-

tention. In addition, he claimed that riboflavin requirement is determined by the oxygen consumption of the body. However, this point is not adequately proved, although worthy of further consideration.

Toi (109) studied the effects of streptomycin and achromycin on the riboflavin content of feces from milk-fed infants to note a decrease in riboflavin after administering 30 mg. per kg. for three days. A larger than normal excretion was obtained five days after the cessation of treatment with the antibiotic.

Clinical observation on the effects of riboflavin supplementation include the work of Maslenikova et al. (110), who confirmed the presence of an increased excretion of riboflavin in patients with severe wounds. As an example, patients with second and third degree burns excreted more than 100 per cent of riboflavin ingested. One may help to explain such observations by noting that during the periods of severe nitrogen loss there is inadequate protein to promote the utilization of riboflavin but that once healing processes become, on balance, greater than the anabolic processes associated with the initial trauma, more than normal amounts of riboflavin are needed to promote maximum cellular growth rates, e.g., protein utilization. In a study of the food intake of patients with tuberculosis, Wilson et al. (111) noted a direct correlation between riboflavin and calcium intake, whether or not milk was consumed. One wonders whether calcium intake can also be related to protein consumed.

Brzezinski et al. (112) showed that the absorption of 150 mg. of riboflavin from a point of injection can be spread out over a period of six weeks if riboflavin is suspended in 2 per cent aluminum monostearate.

The 1958 revision of the National Research Council's *Recommended Dietary Allowances* (63) does not have any appreciable changes in the riboflavin allowance. The ratio of riboflavin allowance to the amount of protein required is retained. However, as the adult requirements for protein have been raised slightly, in recognition of the increased size of present-day Americans, the riboflavin allowance for adults has in effect been increased slightly. Allowances for children and adolescents remain as previously recommended.

Riboflavin in animal nutrition and metabolism.—Bessey et al. (113) studied the riboflavin economy of the rat to show that a rapidly growing rat may require up to three times as much riboflavin as a nongrowing rat. No increase in riboflavin need or of riboflavin destruction in the tissues was noted when metabolism was increased by means of either thyroxin administration or by exposure to a cold environment. The destruction of riboflavin in the rat varied from as little as 0.04 μg. per day in severe deficiency to more than 6 μg. per day when large excesses of riboflavin were administered. This significant paper seems to fortify previously stated hypotheses (114) that manifestations of riboflavin deficiency in man may depend in part upon the accidents of local trauma which may lead to

symptoms such as angular stomatitis or scrotal dermatitis. Thus, one may be quite depleted in riboflavin without showing it if there is little immediate need for tissue repair or for other growth of tissue.

Greenberg & Moon (115) in their studies of the Rhesus monkey interpret recent data to show that plasma riboflavin levels fall more rapidly and give more consistent results than the riboflavin levels of erythrocytes. This is interesting, as it differs from the results reported by Bessey *et al.* (116) on the blood of men in various states of riboflavin nutriture. In the latter study, the erythrocyte riboflavin appeared to be less readily affected by recent riboflavin intake.

An intriguing observation by White & Lincoln (117) on the yellow color of the semen of some bulls showed that the color was caused by high concentrations of riboflavin. They claim that this results from a Mendelian dominant hereditary characteristic that favors the ability of the seminal vesicles to concentrate riboflavin. Studies of effects of riboflavin deficiency on rat tissues included that of Sourkes *et al.* (118), who found significant decreases in the pyrocatechol amine content of the adrenal gland and liver which were not noted during thiamine or pyridoxine deficiency. Kielley (119) noted that the glutamate oxidation by mitochondria from the livers of riboflavin-deficient rats was greatly inhibited by both carcinogenic and noncarcinogenic aminoazo dyes; phosphorylating mechanisms were not affected. Yagi & Okuda (120) studied the riboflavin in the intestinal mucous membranes and found large accumulations of riboflavin and FAD after riboflavin administration. Their studies of the phosphorylating mechanism indicated that phosphorylation of riboflavin can occur by the action of purified intestinal alkaline phosphomonoesterase with β-glycerophosphate as the phosphate donor. They suggest that the phosphorylation of riboflavin *in vivo* may be partly caused by such transferase action in the small intestine.

Investigations by Guerrant & Steel (121) of increased growth rates of rats supplemented with penicillin and chlortetracycline showed no increase in growth rate if the diet was adequate in riboflavin and thiamine. When the diets were inadequate in these two vitamins, there was evidence of better absorption as shown by lower concentration of riboflavin and thiamine in the ceca and feces and higher concentrations in the liver; better utilization was evidenced by lower concentration in the urine.

NICOTINAMIDE

Cellular metabolism.—The biosynthesis of niacin from tryptophan in various species has been summarized by Dalgliesh (122). An excellent review of the metabolic role of nicotinic acid and its participation in the synthesis of pyridine nucleotides has been prepared by Handler (123). Some of the more significant reactions discussed are summarized in Figure 1.

The presence in biological systems of the compound desamido DPN

(nicotinic acid adenine dinucleotide), noted in studies of human erythrocytes by Preiss & Handler (124) and isolated from the mycelium of *Penicillium chrysogenum* by Ballio & Serlupi-Crescenzi (125), has been confirmed by Lamborg *et al.* (126). This nicotinic acid analogue of DPN has been synthesized using beef spleen diphosphopyridine nucleotide (125) and also prepared from the ethyl nicotinate analogue (126).
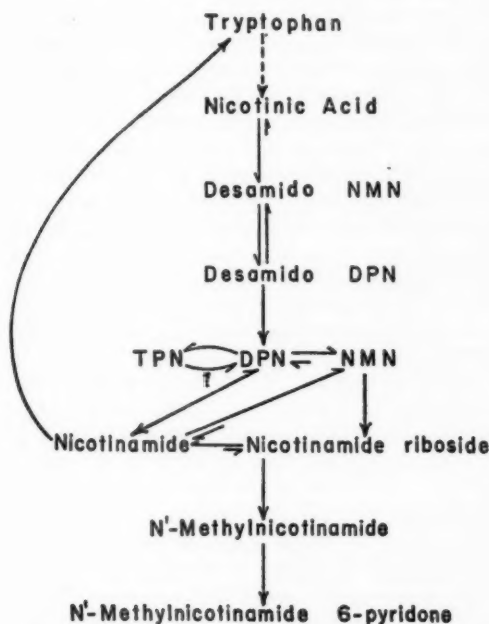
Fig. 1. General scheme of nicotinic acid metabolism [from Handler (123)]

Grossman & Kaplan (128) have purified nicotinamide riboside phosphorylase some eighty fold and noted that it is confined to the cystoplasm of the erythrocyte, whereas the splitting of DPN, TPN, and NMN is localized in the stroma. The enzyme splits nicotinamide riboside either phosphorylytically or arsenolytically and does not appear to have a metal requirement. It is associated with a cofactor (127) capable of inducing nicotinamide sensitivity with the *Neurospora* DPN-ase which is inherently unaffected by free nicotinamide. This cofactor is either ergothioneine or a closely related compound.

Differences in the ability of various species to deamidate nicotinamide have been highlighted by the reports of Sundaram *et al.* (129) and Rajago-

palan *et al.* (130). *Neurospora* does not methylate nicotinic acid but can deamidate nicotinamide. Among the vertebrates, the tissues of the rat, guinea pig, sheep, hog, and cattle cannot deamidate nicotinamide *in vitro,* but the tissues of the pigeon and chick have high nicotinamide deamidase activity. Even in the avian species differences are found; *e.g.,* both the liver and kidney of the pigeon are rich in this enzyme, whereas only the kidney of the chick has such activity.

Hunt *et al.* (131, 132) studied the oxidation of nicotinic acid to 6-hydroxy-nicotinic acid in cell-free extracts of *Pseudomonas fluorescens* and concluded that the oxygen of the hydroxyl group was derived from water. 6-Hydroxynicotinic acid was found to contain the tracer $O_2{}^{18}$ when the latter came from H_2O^{18} but not when the source was gaseous oxygen. Behrman & Stanier (133) reported that the bacterial oxidation of nicotinic acid was catalyzed by a cytochrome-linked, particulate enzyme system. After hydroxylation in the 6-position, an oxidative decarboxylation produced 2,5-dihydroxypyridine which in turn was cleaved oxidatively to yield formic acid and maleamic acid. This is followed by hydrolytic deamination of maleamic acid to maleic acid, which is then isomerized to fumaric acid.

Dietrich *et al.* (134) studied the metabolic action of the nicotinamide antagonist 6-aminonicotinamide to evelute the effectiveness of this compound in experimental neoplasms. They report that it is converted both *in vivo* and *in vitro* to analogues of DPN and TPN. These analogues did not undergo any of the typical addition reactions of normal pyridine nucleotides. McColl *et al.* (135) showed that Walker carcinoma 256 in rats was significantly inhibited by administration of 0.5 mg. per kg. of 6-amino-nicotinamide.

Fischer & Werner (136) reported that solutions of 10^{-7} *M* nicotinamide paralyzed the flagellum of swarm spores of the water mold *Saprolegnia;* when washed free of the nicotinamide they became freely mobile again.

Nicotinamide in human metabolism and nutrition.—The practical implications of recent reports on the utilization of tryptophan as a precursor of nicotinic acid have been highlighted by the substitution of the term "niacin-equivalent" for "niacin" in the 1958 *Recommended Dietary Allowances* of the National Research Council (63). The niacin-equivalent (137) is a compromise based upon studies of the amounts of tryptophan converted to N^1-methyl nicotinamide and its metabolites in human subjects (138, 139). Recent reviews by Goldsmith (140) and by Horwitt (141) have described the experiments conducted and calculations made to confirm the estimation (137) that 60 mg. of tryptophan is the dietary equivalent of 1 mg. of nicotinic acid. Obviously such a relationship can not be inflexible under all conditions of genetic, physiological and dietary variations, but the fact that some protein foods which are virtually devoid of nicotinic acid can supply all the niacin-equivalents necessary for optimum health makes it practical to have some estimation of the amounts of tryptophan in the diet in evalu-

ating nicotinamide requirements. Thus, a diet which provides 70 gm. a day of good protein in the absence of any niacin can be expected to supply more than 720 mg. of tryptophan, the equivalent of 12 mg. of niacin, from the protein component alone. Another interesting point that has come out of comparisons of past and present studies of human requirements is the calculation that the requirement for niacin-equivalent is dependent upon total caloric intake, either as a function of metabolism plus work (138) or of body size (140). Accordingly, it may be necessary to think of the requirements for nicotinamide-tryptophan, as one does for thiamine requirements, as being related to calories consumed. Since calculations of niacin-equivalents per 1000 Cal., sometimes called the niacin ratio (138, 141), have indicated that 4.4 niacin-equivalents per 1000 Cal. is the minimum necessary to prevent pellagra in man, the new allowances (63) have taken a niacin ratio of 4.4 as a base line, to which a safety factor of approximately 50 per cent has been added to give an allowance of 17 niacin-equivalents per 2500 Cal. In effect, this has decreased previous estimations of nicotinic acid requirements.

Recent corroboration of the 60 to 1 ratio used in calculating niacin-equivalents has been obtained from the studies of Wertz et al. (142), who reported that the amount of tryptophan equivalent to 1 mg. of nicotinic acid varied from 45 to 69 mg. in four female subjects. Of interest is their confirmation of earlier observations that N^1-methyl nicotinamide is markedly increased in the third trimester of pregnancy which, in effect, produced a smaller tryptophan to nicotinic acid ratio of 18 to 1 in these women. This points up the difficulties that may be encountered in evaluating the urinary levels of vitamins and their metabolites during periods of protein catabolism.

Morley & Storvick (143) studied the blood and urine content of nicotinic acid metabolites in four women under controlled dietary conditions for a 30-day period. The diet provided 8.7 mg. of nicotinamide and 770 mg. of tryptophan from 60 gm. of protein. The mean excretions of N^1-methyl nicotinamide and its 6-pyridone were 5.8 and 7.3 mg. per day, respectively, amounts which testify to the complete adequacy of the 21 niacin-equivalents fed.

Banerjee & Agarwal (144) have claimed differences in the urinary excretion of products of tryptophan and nicotinic acid metabolism between normal subjects and others suffering from various diseases, but the data reported are difficult to interpret for lack of complete information about ingestion of these compounds prior to the experimental period. The same criticism applies to many papers which claim marked alterations in the excretion of the products of tryptophan metabolism by patients with schizophrenia (145).

The efficacy of nicotinic acid administration in reducing serum cholesterol has been the subject of numerous recent reports (146 to 151). Altschul & Hoffer (152) have shown that such decreases in serum cholesterol are

accompanied by an increase in basal metabolism in normal young adults. The mechanism by which nicotinic acid accomplishes this decrease in blood cholesterol is not yet resolved; but since nicotinamide has no such effect, one wonders whether the stresses of detoxifying the large doses of nicotinic acid used (3 gm. per day) are at least partially responsible for the decreased cholesterol synthesis, since many nonspecific stresses lower the cholesterol level of the blood.

Sydenstricker (153) has published an interesting historical review on the history and conquest of pellagra.

Nicotinamide metabolism and nutrition in animals and birds.—Studies by Chalpoupka *et al.* (154) of the relative roles of nicotinic acid and tryptophan in maintaining the blood PN in rats have confirmed previous reports by Williams *et al.* (155) that physiological levels of tryptophan were more active than niacin in stimulating synthesis of rat liver pyridine nucleotides in animals previously depleted of liver PN.

The lowering of blood cholesterol levels in rats by giving large doses of nicotinic acid has been investigated by Schön (156), who showed that liver cholesterol decreased as the nicotinic acid in the diet was raised from 1 to 4 per cent. These results were attributed to the special demand for methyl groups to detoxicate nicotinic acid causing a relatively decreased synthesis of cholesterol.

Ranke *et al.* (157), working with rats, have claimed that B_{12} deficiency impairs the ability of the liver to methylate nicotinamide. Horger & Gerheim (158) investigated the adverse effect on growth of 0.4 per cent nicotinamide in rat diets. The addition of 2 per cent methionine to a diet containing only 0.1 per cent nicotinamide also inhibited growth, but it is interesting that the addition of 1 per cent malic acid to this diet prevented this growth inhibition.

The effect of alkali-treatment of corn upon making its nicotinic acid content more available to animals was studied by McDaniel & Hundley (159). Littermates of weanling puppies which developed blacktongue in 67 to 120 days on untreated corn gained considerably more weight and did not show symptoms of deficiency after 300 days on the diet. Chicks fed treated corn gained an average of 170 gm. in 30 days as compared to 45 gm. on untreated corn. However, rats did not show differences in growth as a result of alkali-treatment of corn. This was attributed to more efficient conversion of tryptophan to nicotinic acid by rats than by the other two species.

Van Reen & Stolzenbach (160) studied the effects of various pyridine derivatives as growth factors in the diet of ducklings. Pyridyl-3-aldehyde increased growth as much as nicotinamide. Pyridyl-3-carbinol and β-picoline were relatively ineffective when incorporated into the diet, but both showed a positive response when administered *per os* twice a week. Given intraperitoneally, all three compounds were effective, and β-picoline appeared to be a better precursor for DPN than nicotinic acid.

Chang & Johnson (161) used C^{14}-carboxyl-labeled nicotinamide to study metabolic excretion patterns of nicotinic acid in chicks. Metabolites identified were β-nicotinyl-D-glucuronic acid, nicotinuric acid, δ-nicotinyl ornithine, α-nicotinyl ornithine, and 2,5-dinicotinyl-ornithine in addition to nicotinic acid and nicotinamide.

PANTOTHENIC ACID

Biosynthesis, chemistry, and methods.—The biosynthesis of panthothenic acid in microorganisms has been reviewed by Maas (162). Pantothenate is one of the few substances produced by organisms like *E. coli* in large excess of their needs and excreted into the surrounding medium. This is probably the reason pantothenate deficiency has not been a nutritional problem in man; his intestinal tract normally harbors a considerable concentration of *E. coli*. Another review, Novelli (163), supplies additional information on the turnover of pantothenate in mammalian organisms. This review is recommended as a source of information on the biosynthesis and enzymatic degradation of CoA. Mammals are apparently unable to catabolize pantothenate as they lack the enzyme needed to break the peptidic bond; excesses in the circulation are largely excreted intact.

A series of papers from Ochoa's laboratory (164 to 167) which report on the metabolism of propionic acid provides well-organized examples of CoA functions and techniques of investigating this compound. McMurray & Lardy (168), working with submitochondrial particles from sonic extracts of rat liver mitochondria, have reported on the role of CoA in phosphorylations associated with electron transport. Purified CoA markedly stimulated phosphate uptake without affecting oxygen consumption when D,L-β-hydroxybutyrate was the substrate. Addition of CoA stimulated oxidative phosphorylation when D(-)β-hydroxybutyrate, ethanol plus yeast alcohol dehydrogenase, DPNH, or succinate was used as substrate. Only reduced CoA promoted phosphorylation.

Airth *et al.* (169) reported on the activity of CoA in stimulating firefly luciferin as follows: Firefly luciferin ($C_{13}H_{12}N_2S_2O_3$) reacts with ATP to form pyrophosphate and active luciferin, which is probably adenyl luciferin. Light emission results from the oxidation of active luciferin to adenyl oxyluciferin ($C_{13}H_{10}N_2S_2O_3$), which eventually decomposes to adenylic acid and oxyluciferin. CoA removes oxyluciferin from the enzyme surface to form oxyluciferyl-CoA, thus stimulating light emission. Oxyluciferyl-CoA can react with cystine, glutathione, or hydroxylamine nonenzymatically to form corresponding oxyluciferin derivatives. In the presence of luciferase, oxyluciferyl-CoA can be split by adenylic acid; and when excess pyrophosphate is added, ATP and free oxyluciferin are formed. The incorporation of C^{14}-adenylic acid into ATP depended upon the presence of CoA in the reaction mixtures. The determination of CoA in animal tissues by releasing pantetheine enzymatically, followed by microbiological

estimation of pantetheine and free pantothenic acid, was studied by Wolff et al. (170). Both of these factors exert the same effect on *L. arabinosis* in the presence of cysteine and in an atmosphere of CO_2 (171). Intestinal phosphatase was employed to split the CoA. A similar technique using *L. casei*, also a modification of the method of Novelli et al. (172), was reported by Clegg (173) to show that flour (70 per cent extraction) contained about half the total pantothenic acid content of the original whole wheat.

Pantothenic acid metabolism and nutrition.—Since the hepatic concentration of CoA is diminished in methionine deficiency (174) and ethionine has been shown to interfere with the metabolism of methionine, Wenneker & Recant (175) studied the effect of prolonged ethionine feeding on the coenzyme content of rat liver and kidneys. The total CoA concentration and the hepatic CoA content were decreased in the ethionine-fed rats as compared with the pair- and *ad libitum*-fed controls, but the renal CoA of the ethionine-fed rats was increased over that of the pair-fed controls.

Barboriak & Krehl (176) reported on the influence of ascorbic acid, sulfathiazole, and the antibiotic hygromycin on effects of pantothenic acid deficiency in rats. Either ascorbic acid or hygromycin delayed or suppressed the appearance of deficiency signs, but sulfathiazole, fed simultaneously with ascorbic acid, counteracted its beneficial effect. This substantiates current opinions that the favorable effects of ascorbic acid when used in relatively large amounts (2 per cent) are mediated through changes in the intestinal flora. The influence of chlorotetracycline on the pantothenic acid requirement of young pigs was studied by McKigney et al. (177). Although gross deficiency symptoms were absent in the pigs given 10 mg. per lb. of this antibiotic, the concentrations of pantothenic acid in the brain, heart, and kidney were not increased.

Cooperstein & Lazarow (178) studied the CoA as a possible site of action of alloxan, after reasoning that since destructive effects of alloxan on the β-cells of the islands of Langerhans are inhibited by prior injections of glutathione or cysteine, the sulfhydryl group of CoA might be involved. Using the pigeon liver acetylating system, alloxan at a concentration of 5×10^{-4} M produced a 23 per cent inactivation of CoA and a 95 per cent inhibition of the liver acetylating enzyme complex. These inactivations were partly reversed by the addition of cysteine.

The place of pantothenic acid in the physiology of stress and the relationship of adrenocortical compounds to this stress continues to receive attention. Langwill et al. (179) report that the production of corticosterone is reduced in rat adrenals during pantothenate deficiency, which partially confirms the recent reports of others that such deficiency decreased adrenocortical secretion [Eisenstein (180), Fidanza et al. (181), Olivi et al. (182)]. Weiss (183) has used psychobiological techniques to show that rats deprived of pantothenic acid are more sensitive to cold than animals supplemented

with this vitamin. Zucker (184) reviewed the effects of pantothenic acid deficiency on the integrity of the intestine with emphasis on the production of duodenal ulcers in pantothenic acid-deficient rats.

The reciprocal relationship between B_{12} and pantothenic acid has been confirmed by several workers. Radhakrishnamurty & Sarma (185) found that the vitamin B_{12} content of livers of pantothenic acid-deficient rats was double that of the supplemented controls. Balloun & Phillips (186) report that B_{12} has a sparing action on the level of pantothenic acid required by chicks, and Moruzzi et al. (187) report a striking increase in the liver content of pantothenic acid and CoA of rats on a vitamin B_{12}-deficient diet.

The difficulties in evaluating human studies on pantothenic acid continue to plague interpretations of human requirements, and one must rely upon extrapolations from animal experiments. Barboriak et al. (188) have estimated that the adult rat requires between 0.8 to 1.0 mg. of calcium pantothenate per 100 gm. of diet. This is based upon ability to acetylate injected sulfonamide. In another study by Barboriak et al. (189) which depended upon the effect of suboptimal levels of pantothenate on the reproductive performance of the rat, 0.2 mg. per 100 gm. diet was found to produce serious anatomical impairment of the testes, and in female rats decreased fertility and litter resorption were noted at levels of 0.5 mg. per 100 gm. of diet. The growth capacity of the rat is apparently not affected adversely by pantothenic acid deficiency, as even after a relatively long period of severe deficiency, pantothenate can cause growth resumption. After short-term deficiencies, in order to obtain optimum growth rates, as much as 1.2 to 2.0 mg. per 100 gm. of diet are needed (190). However, these animals may develop permanent changes of the adrenal glands as a consequence of the short-term deficiency of pantothenic acid.

LIPOIC ACID (THIOCTIC ACID)

Both Gunsalus (191, 192) and Reed (193) have recently reviewed the chemistry and function of lipoic acid with emphasis on its participation in enzymatically catalyzed reaction. That lipoic acid may serve as an acyl acceptor has been demonstrated by isolation of acetyl and succinyl thioesters (191). Acyl (acetyl, succinyl, and phosphoryl) transfer reactions of mercapto and phospho compounds, implicated in activation, energy transfer, and biosynthetic reactions, have been described (192).

Evidence has accumulated (194) to show that lipoic acid in a protein-bound form is involved in the oxidative decarboxylation of α keto acids as follows:

$$RCOCO_2H + HS{-}CoA + DPN^+ \xrightarrow{\text{TPP}} RCO{-}S{-}CoA + CO_2 DPNH + H^+$$
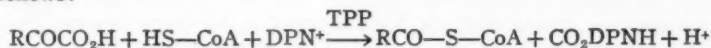
Reed & De Busk (195) had previously proposed that the lipoyl moiety is attached to TPP through an amide linkage, but this is not supported by newer evidence presented by Reed et al. (194) that TPP is not required for

incorporation of radioactive lipoic acid into the *Streptococcus faecalis* apopyruvate dehydrogenation system. The nature of the protein-bound lipoic acid was studied (196), and it was found that it could be released from the protein by an enzyme, "lipoyl-X hydrolase," obtained from *S. faecalis*. The binding of the lipoic acid to apoenzyme involves two enzymatic reactions: (*a*) an ATP-dependent activation of lipoic acid forming lipoyl adenylate, and (*b*) a transfer of the lipoyl moiety to apoenzyme. Reed *et al.* (196) have also presented evidence that the dihydrolipoic transacetylase and dihydolipoic dehydrogenase are not mediated through protein-bound lipoic acid. These enzymes appear to react directly with the free dithiols.

Sanadi & Searls' study (197) of the α-ketogutaric dehydrogenase complex from hog hearts showed that it could catalyze the reversible oxidation of reduced DPN by 6,8-thioctate and 6,8-thioctamide. (+)-Thioctate was active and (−)-thioctate was inactive in this reaction.

Reiss & Hellerman (198), working with rat heart sarcosomes, have reported that the inhibition of pyruvate utilization by an arsenoso compound, α-(*p*-arsenosophenyl)-butyrate, can be reversed by lipoic acid. Since this reversal is affected by both the oxidized and reduced forms of lipoic acid and not by cysteine, they believe this to mean that lipoic acid may have a functional role in the pyruvate metabolism of rat heart mitochondria.

Acker & Wayne (199) have studied methods of synthesizing racemic, optically active, and radioactive lipoic acids using the reaction of 6,8-dichloro-octanoic acid or its esters with sodium sulfide.

In evaluating the effects of lipoic acid in biological systems, it is often difficult to distinguish between what might be a nonspecific antioxidant effect and what might be directly involved in cellular enzymatic processes. In this respect, physiological investigations of lipoic acid are somewhat reminiscent of research in the function of vitamin E. Rosenberg & Culik (200) have reported that lipoic acid, which has a strong redox potential, can substitute for much of the ascorbic acid in the diet of guinea pigs and that 15 mg. of lipoic acid per kg. of diet brought about reproduction as effectively as 25 mg. of α-tocopherol, as measured by rate of conception and number of young born and weaned. Kofler *et al.* (201) studied the protective action of lipoic acid against x-rays in rats to show that 120 mg. per kg. injected intraperitoneally gave a high incidence of survival to a lethal dose of irradiation. Similarly, Manca & Asole (202) reported that in the presence of ferricyanide, *p*-aminophenol chromate, or sodium dichromate, lipoic acid has an antimethemoglobinizing action. However, Eger (203) showed that the liver protective action of lipoic acid in the allyl alcohol test was less than that obtained with cysteine. Lipoic acid has been reported to be more effective than β-mercaptoethylamine in protecting mice (Genazzani *et al.* (204)) against x-irradiation.

Claims of action of lipoic acid in alleviating hepatic disorders (205, 206, 207) are especially difficult to evaluate in view of the known effects of

cysteine in modifying certain types of fatty livers. Paterni *et al.* (208) have shown that lipoic acid has a favorable influence on the length of life and fatty degeneration of rats poisoned with carbon tetrachloride.

Sheffner & Adachi (209) reported that the stimulation of *S. faecalis* growth by lipoic acid was obtained only when the concentrations of iso-leucine,valine, and to a lesser extent arginine were suboptimal for maximum growth. No stimulation by lipoic acid was observed in media deficient in other amino acids or in media containing adequate amounts of all the essential amino acids.

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WATER-SOLUBLE VITAMINS, PART II[1,2]

(Vitamin B_{12}, Folic Acid, Ascorbic Acid, Biotin, Vitamin B_6, Miscellaneous)

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Limitations of space have necessitated a selective approach in this year's review. In an attempt to do justice to topics that have aroused considerable interest during the past year, certain aspects normally discussed in these articles have been treated only in outline or omitted.

Vitamin B_{12}

Chemistry and biosynthesis of vitamin B_{12} and its analogues.—The chemical structure of vitamin B_{12} and many of its naturally occurring analogues is now firmly established and interest is focused on possible pathways in their biosynthesis. The question has been reviewed by Kon & Pawelkiewicz (1), who put forward a tentative scheme for the biosynthetic formation of cyanocobalamin and its analogues. Work by Juillard (2) with a mutant of *Bacillus megaterium* which synthesized the monocarboxylic acid of Factor B led him to suggest that this compound was an intermediate in the biosynthesis of cobalamin. Pawelkiewicz & Zodrow (3) consider it more likely to be a breakdown product, since it appeared late during the fermentation of *Corynebacterium diphtheriae*. Its appearance was preceded by that of two unidentified phosphoric esters of Factor B and lent support to the earlier suggestion by Dellweg, Becher & Bernhauer (4) that phosphorylation of Factor B was a step in the formation of cyanocobalamin. Factor B monophosphate has been isolated by Di Marco, Boretti, Migliacci, Julita & Minghetti (5) during the growth of a strain of *Nocardia*, in addition to another compound identified chemically as a guanosine diphosphoric ester

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[1] The survey of the literature pertaining to this review was concluded in October 1958. One or two papers available to the authors in proof were included for the sake of completeness.

[2] The following abbreviations are used: ACTH for adrenocorticotropin; ATP for adenosine triphosphate; DNA for deoxyribonucleic acid; DPNH for diphosphopyridine nucleotide (reduced form); FAD for flavin-adenine-dinucleotide; INH for isonicotinic acid hydrazide; PAB for *p*-aminobenzoic acid; PABG for *p*-aminobenzoylglutamic acid; PALP for pyridoxal phosphate; PAMP for pyridoxamine phosphate; PGA for pteroylglutamic acid; PGAH$_2$ for dihydropteroylglutamic acid; PGAH$_4$ for tetrahydropteroylglutamic acid; RNA for ribonucleic acid; SRNA for supernatant ribonucleic acid; TPNH for triphosphopyridine nucleotide (reduced form).

of Factor B. Chemical and enzymatic studies suggested the following structure:

$$\text{Factor B}\underset{\underset{\parallel}{O}}{\overset{\overset{O^-}{|}}{P}}-O-\underset{\underset{\parallel}{O}}{\overset{\overset{O^-}{|}}{P}}\text{—ribose guanine}$$
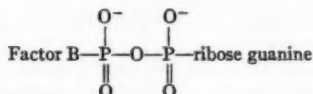
Guanosine nucleotides can function as coenzymes in some reactions involving formation of guanosine diphosphomannose followed by pyrophosphorolysis giving rise to guanosine triphosphate and mannose-1-phosphate. By analogy the authors suggest that their guanosine diphosphoric ester of Factor B may be an intermediate in the formation of Factor B monophosphate, which might then be utilized in the synthesis of cyanocobalamin.

The monomethylbenziminazole derivative of cyanocobalamin has hitherto only been prepared as a mixture of the 5– and 6– methyl derivatives. Bernhauer & Friedrich (6) have recently separated chromatographically, with subsequent chemical and physical identification, the pure 5– and 6– methyl derivatives from a culture of *Propionibacterium shermanii*. The biological activity of these analogues is being investigated and should provide useful information regarding the relative importance to biochemical function of the 5– and 6– positions. Some new analogues have been produced by guided synthesis, with *Propionibacterium arabinosum,* by Perlman & Barrett (7). These were not obtained in crystalline form, but on the basis of chromatographic and ionophoretic evidence the authors claim to have replaced dimethylbenziminazole in the cyanocobalamin nucleotide with a phenazine, a quinoxaline, a quinazoline, a benztriazole or a benzthiazole grouping. All the resulting analogues had growth-promoting activity for *Ochromonas malhamensis* as well as for *Escherichia coli* and *Lactobacillus leichmannii*. It would be of interest to have the structure of these compounds verified chemically, since so far the only analogues to show marked activity for *O. malhamensis* have contained benziminazole or its derivatives in the nucleotide.

Metabolic role of vitamin B_{12}.—Present knowledge of the metabolic role of vitamin B_{12} was recently reviewed by Lester Smith (8), who speculates on the nature of the molecular groupings that may be involved in its biochemical functions, and by Arnstein (9), who includes among the metabolic reactions known to involve vitamin B_{12} (*a*) methyl group synthesis, (*b*) deoxyriboside synthesis, (*c*) activation of sulphydryl enzymes, and (*d*) protein synthesis.

Although it is now generally accepted that vitamin B_{12} functions in the biosynthesis of labile methyl groups from 1-carbon precursors, it is not known at which step in the synthesis the vitamin is required. Arnstein (10) compared the metabolism of formate and histidine in rats depleted of vitamin B_{12} and in nondepleted rats. The increase in incorporation of

[14]C-formate into choline was three times as great in the animals receiving vitamin B_{12}, but the incorporation of 2-^{14}C-histidine, a formate precursor, was not affected by the deficiency, indicating that free formate is probably not an intermediate in the formation of methyl groups from histidine. The author suggests a possible function of vitamin B_{12} in an oxidation-reduction system related to the metabolism of formate. Formate and other precursors of DNA-thymine have been studied by Dinning, Allen, Young & Day (11) with *L. leichmannii* grown in the presence of deoxycytidine. Additions of vitamin B_{12} to the medium resulted in a fivefold increase in the incorporation of ^{14}C-formate into DNA-thymine, but incorporation of α-^{14}C-glycine, β-^{14}C-serine, or ^{14}C-Me-methionine was not affected. These results are interpreted as indicating that vitamin B_{12} is necessary for reduction of formate to thymine methyl by a pathway that does not involve methionine or hydroxymethyl intermediates. Vohra, Lantz & Kratzer (12) allowed ^{14}C-formaldehyde to react *in vitro* with vitamin B_{12}. Complexes containing one-half, one, two, or three molecules of formaldehyde to one of vitamin B_{12} were formed, and the authors suggest, by analogy with hydroxymethyltetrahydrofolic acid, that the ability of vitamin B_{12} to form complexes with formaldehyde may have some biological significance.

Studies on the relation between vitamin B_{12} and deoxyribonucleic acids in microorganisms have been continued. In a vitamin B_{12}-requiring mutant of *E. coli*, Wacker & Pfahl (13) showed that no deoxyriboside was formed from riboside in either the absence or presence of vitamin B_{12}. In experiments with two strains of *L. leichmannii*, Wacker, Pfahl & Schröder (14) found that replacement of deoxyribosides by vitamin B_{12} in the medium resulted in a three- or fourfold increase in DNA per cell, whereas RNA concentration was unaltered. In a brief communication, Wacker, Kirschfeld & Träger (15) state that a parallel can be drawn between the clinical activity of several vitamin B_{12} analogues and their effect on DNA synthesis by *L. leichmannii* 313 and suggest using this reaction as a possible assay for the clinical activity of vitamin B_{12}-like compounds.

Earlier claims that vitamin B_{12} might be concerned in nucleic acid metabolism in animals have not received much substantiation. Wagle, Vaughan, Mistry & Johnson (16) measured the incorporation of ^{14}C-labelled 1-carbon metabolites into nucleic acids in normal and vitamin B_{12}-deficient rats and pigs. The authors state that vitamin B_{12} did not affect incorporation of any of the precursors into nucleic acids, but a close inspection of their results reveals small increases of ^{14}C activity in the liver RNA of animals given vitamin B_{12} when formate, formaldehyde, glycine, or serine were used as precursors. These increases may not have been significant, but might be worthy of further investigation.

The relation between vitamin B_{12} and sulphydryl groups has not been clarified by recent work. In thyroid glands from vitamin B_{12}-deficient hens and chick embryos, Ferguson *et al.* (17) noted a marked reduction in

content of sulphydryl groups. They have also observed lowered glutathione levels in the blood of vitamin B_{12}-deficient chicks. Jaffé (18) found a lowered liver glutathione in vitamin B_{12}-deficient mice only in fasting animals or in those with a low intake of S-containing amino- acids. Cysteine, cystine, and methionine were all as effective as vitamin B_{12} in restoring liver sulphydryl levels in mice given protein-free diets. Thus it seems possible that the effects of vitamin B_{12} on liver glutathione may be secondary to its role in methionine synthesis. Earlier evidence by Chow and his colleagues suggesting possible involvement of vitamin B_{12} in carbohydrate metabolism has been strengthened by the finding of Chang, Davis, Hsu & Chow (19) that deprivation of vitamin B_{12} led to increased pyruvic and lactic acids in the blood of rats. This result might be an indirect effect of a fall in glutathione, known to be concerned in glycolysis. Hsu, Okuda, McCollum & Chow (20) have found increased amounts of vitamin B_{12} in the livers of rats deprived of pantothenic acid, adding further evidence of a possible relation between vitamin B_{12} and pantothenic acid.

The most discussed hypothesis regarding the possible biochemical function of vitamin B_{12} is the suggestion put forward last year by Johnson and his collaborators that vitamin B_{12} may be concerned in protein biosynthesis. The findings (21) that the incorporation of amino acids into protein by microsomal preparations from deficient rats was less than in those from normal animals and that injected 3-^{14}C-serine was less well incorporated into liver proteins of vitamin B_{12}-deficient pigs and rats (22) were followed by further experiments *in vivo* and *in vitro*. All the *in vivo* studies (23) resulted in a lower incorporation of amino acids into liver proteins of vitamin B_{12}-deficient rats and pigs than in the corresponding normal animals. Injection of ^{60}Co-labelled vitamin B_{12} into a rat and subsequent analysis of liver fractions (24) showed most of the label in the cell supernatant was present in the "pH 5 enzyme," concerned with activation of aminoacids. The authors thereafter termed this fraction the "B_{12}-enzyme." Preparations of the enzyme from deficient rats had little effect on the incorporation of amino acids by microsomes from normal controls, whereas the enzyme from normal animals increased threefold the incorporation by microsomes from the deficient rats. It was therefore postulated that the B_{12} enzyme acted as an "activator-carrier" for amino acids to the template and that such a role might also explain the function of vitamin B_{12} in methyl synthesis, where *possibly* it is involved in the activation of glycine, serine, and formate. In later experiments (25, 26) most of the activity of the pH 5 enzyme was found in the fraction precipitated by 40 to 60 per cent ammonium sulphate. On incubation with ATP-^{32}P-pyrophosphate a large amount of radioactivity was incorporated into the ATP; this suggested that vitamin B_{12} functions in the activation reaction:

$$\text{amino acid} + \text{ATP} \xrightleftharpoons{\text{B}_{12}\text{ enzyme}} \sim \text{amino acid} + \text{PP}$$
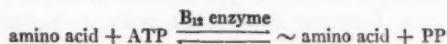
A decrease of ^{32}P incorporation into ATP in the presence of an antivitamin B_{12} compound (the anilide of the monocarboxylic acids derived from cyanocobalamin) was partially restored by increasing the concentration of B_{12}-enzyme.

In preliminary *in vivo* experiments Holdsworth (27) found no difference in amounts of ^{14}C-labelled glycine, serine, or hydrolyzed *Chlorella* protein incorporated into liver protein of vitamin B_{12}-deficient rats or chicks. Very recent work by Fraser & Holdsworth (28) with minces and subcellular fractions from chicks has also failed to confirm the Illinois findings, although the experimental conditions were slightly different. In both normal and deficient chicks Fraser & Holdsworth found most of an injected dose of ^{60}Co-vitamin B_{12} in the pH 5 supernatant rather than the pH 5 enzyme and questioned the particular association of vitamin B_{12} with the amino acid-activating enzyme. Further, the rate of amino acid activation, as measured by the hydroxamate test (29) or by incorporation of amino acids into SRNA, was not decreased but increased in the deficient birds. ATP-^{32}PP exchange was not noticeably different in preparations from normal and deficient birds and, although the exchange reaction was depressed by high concentrations of the vitamin B_{12} antagonist and partly restored by further addition of the vitamin, this effect was independent of the presence of amino acids in the incubation mixture and may not have been a true measure of amino acid activation. These authors suggest that some small and inconsistent effects which they observed in liver minces and some microsomal preparations may reflect an indirect role of vitamin B_{12} in protein biosynthesis.

Arnstein & Simkin (30) have also attempted to repeat the work of Wagle *et al.*, using minces and cell-free preparations from normal and vitamin B_{12}-deficient rat livers. In liver minces from deficient animals incorporation of amino acids into protein was less than in the controls but was not appreciably stimulated by addition of vitamin B_{12}. Similarly, vitamin B_{12} did not stimulate incorporation of uniformly labelled ^{14}C-L-phenylalanine into microsomal or cell-sap protein. The authors do not deny the possibility that vitamin B_{12} deficiency may result in decreased protein synthesis, but suggest that the effect is secondary to some other biochemical function of the vitamin.

If an uncomplicated deficiency of vitamin B_{12} in the experimental animals used is assumed, these opposing sets of results are difficult to explain. Both Arnstein & Simkin (30) and Fraser & Holdsworth (28) checked the state of depletion of their animals by determination of liver levels of vitamin B_{12}. The statement that haemoglobin was lowered in the deficient animals used by Wagle and his colleagues is noteworthy, since anaemia has not been frequently produced in animals deprived of vitamin B_{12}. In any vitamin deficiency the demonstration of a metabolic difference between normal and depleted animals does not necessarily imply direct involvement of the vitamin in the particular process studied. If the effect of vitamin

B_{12} on protein biosynthesis is only indirect, small differences in composition of the isolated preparations used could account for lack of agreement in results of *in vitro* experiments between different groups of workers. If, as Lester Smith (8) suggests, a vitamin B_{12}-protein complex rather than the free vitamin is the biologically active form, failure to produce an effect on addition of crystalline vitamin B_{12} is not surprising and may simply reflect the absence of the appropriate binding substance from the system. In this connection a vitamin B_{12} peptide from liver was no more effective than the free vitamin in the experiments of Fraser & Holdsworth (28).

If vitamin B_{12} is directly concerned in protein biosynthesis, many of the functions postulated for this vitamin can be accounted for as secondary effects of reduced enzyme formation. Conversely, it is surprising that deprivation of vitamin B_{12} does not lead to rapid failure in lactation in mammals or to reduced egg production in birds, since these are among the first consequences of amino acid insufficiency. There is still reason for speculation that the findings of Wagle and his colleagues regarding vitamin B_{12} and protein synthesis are secondary to other functions postulated for vitamin B_{12}. For instance, an effect on sulphydryl balance could be reflected in the many reactions for which glutathione is required, including the activation of amino acids. Similar results might follow impaired carbohydrate metabolism.

Absorption of vitamin B_{12}: function of intrinsic factor.—Attempts to isolate and characterize intrinsic factor continue (31, 32). So far, the most potent preparation has been prepared by Williams & Ellenbogen (33). Their material is clinically active, as measured by the Schilling (34) test, at a level of only 0.3 mg., and has a molecular weight between 5000 and 10,000. Studies of the mechanism of absorption of vitamin B_{12} are hampered by lack of a pure intrinsic factor, and it is difficult to determine which of the properties attributed to vitamin B_{12}-intrinsic factor complexes are directly concerned with absorption of the vitamin and which are side effects of accompanying impurities. For example, the relation of binding to the physiological action of intrinsic factor is still unsolved, and so is the question of its possible species specificity. Gräsbeck (35), in a study of the effects of agents which blocked specific groups in vitamin B_{12}-intrinsic factor complexes, found that whenever the treatment resulted in a loss of vitamin B_{12} binding capacity there was also a decrease in intrinsic factor activity. Wolff & Vuillemin (36) reported that urea did not inhibit the capacity of a gastric mucosal extract to combine with vitamin B_{12}; certain saponins abolished this capacity, but only if they were added to the extract before the vitamin. Certain binding systems appear to show a preference for vitamin B_{12} in the presence of its analogues (37, 38, 39). Such a mechanism might explain the relatively poor absorption of pseudovitamin B_{12} and other analogues, compared with that of vitamin B_{12} itself.

Taylor & Morton (40) have produced antibodies to both human and pig intrinsic factor preparations by injection of extracts of human gastric mucosa or pig pylorus into rabbits. Human and pig intrinsic factor activity

in pernicious anaemia patients was specifically inhibited by the appropriate rabbit antiserum. The authors suggest that the ability of the rabbit to produce antibodies to these preparations may indicate that intrinsic factor is wholly or partly species specific, or may simply be the result of sensitization following parenteral administration of what is normally an external secretion. An intrinsic factor-inhibiting substance has been reported by Schwartz (41) in the serum of pernicious anaemia patients treated orally for long periods with vitamin B_{12} plus pig pyloric mucosa. This substance was not present in the serum of untreated patients or of healthy subjects. Possibly Schwartz's inhibiting substance is analogous with those produced experimentally by Taylor & Morton (40), but, as both groups were working with relatively crude preparations, it is impossible to know whether the antigen was intrinsic factor itself, or accompanying protein also concerned in the absorption of vitamin B_{12}.

A new concept regarding the absorption of vitamin B_{12} has recently been put forward by Heathcote & Mooney (42, 43), who point out that vitamin B_{12} is usually present in food in a bound form not assimilable until proteolyzed to what they suggest may be a dialyzable vitamin B_{12}-peptide complex. They claim to have produced such a complex by fermentation of a *Streptomyces* mutant and consider it the most effective oral preparation yet described for the treatment of pernicious anaemia. These claims have not been entirely accepted on the grounds that the amount of vitamin B_{12} peptide given in the initial stages of their clinical trials was high enough to have brought about remission by its vitamin B_{12} content alone (44, 45) and, further, that insufficient proof was offered that the material tested was a true complex rather than a simple mixture of free vitamin B_{12} and peptide (46). The hypothesis that pernicious anaemia may be the result of an impaired proteolysis has been severely questioned by Glass (47), since it cannot be reconciled with much available information on digestive processes in pernicious anaemia and other gastrointestinal disorders. Nor can the suggestion of Heathcote & Mooney (42) that intrinsic factor probably does not exist be accepted without discounting past evidence that absorption of vitamin B_{12} is promoted by substances produced in the gastrointestinal tract, for which further evidence has accumulated. Wolff (48) reported that an extract of rat gastric mucosa promoted the fixation of ^{60}Co-vitamin B_{12} to the wall of the rat small intestine. Coates & Holdsworth (49) found that rat stomach extract increased, and pig intrinsic factor decreased, uptake of labelled vitamin B_{12} by the small intestine of the live rat and by isolated cell preparations of rat intestinal mucosa. Clayton, Latner & Schofield (50) increased absorption of vitamin B_{12} in gastrectomized rats by administration of rat gastric juice. Many results point to the ileum as the most active site of absorption of vitamin B_{12} (39, 48, 49, 51), and it seems likely that if an "acceptor factor" is necessary for the transport of vitamin B_{12} across the wall of the gut (52) it may well be concentrated in the cells of the ileal region.

It is becoming more apparent that intrinsic factor plays a role, not only in the absorption of vitamin B_{12} from the gastrointestinal tract, but also in its retention by the tissues. Pig intrinsic factor increased the uptake of vitamin B_{12} by liver slices and rat small intestine (53) and by liver homogenates (54). Materials with intrinsic factor activity were reported by Miller (55) to enhance the vitamin B_{12}-combining power of serum, which was associated with the $alpha_2$ and beta globulins, or materials having the same electrophoretic mobility. It is interesting that in all these experiments the tissue uptake of vitamin B_{12} was enhanced by intrinsic factor preparations from different sources and not necessarily prepared from the same species as the experimental tissue. In our own work with rats (56), rat gastric extracts aided retention of vitamin B_{12} by the animal, whereas vitamin B_{12} given with clinically active intrinsic factor preparations from other species was much more rapidly excreted. A possible *in vitro* method of measurement of intrinsic factor, based on its enhancement of uptake of vitamin B_{12} by liver slices, has been proposed by Herbert (57), and Miller (55) has suggested that the vitamin B_{12}-combining reaction of serum might also be adapted for an assay.

Considerable interest has been aroused by the announcement of Chow, Meier & Free (58) that D-sorbitol enhanced the absorption of vitamin B_{12} in clinically healthy men. A similar effect of D-sorbitol was observed in rats by Greenberg, Herndon, Rice, Parmelee, Gulesich & Van Loon (59) who extended their studies to other carbohydrates. They found that D-mannitol, L-sorbose, and D-xylose similarly enhanced absorption of vitamin B_{12}, whereas sucrose, D-galactose, D-glucose, D-fructose, and D-mannose had no such effect. Barnard (60) has drawn attention to previous work in which vitamin B_{12} given by mouth with a sorbitan monooleate polyoxyethylene derivative (Tween 80) or with D-mannitol was effective in megaloblastic anaemia. Since D-sorbitol is the first substance other than intrinsic factor to enhance uptake of vitamin B_{12}, its mode of action is of much interest. Schilling (61) suggests that the action of sorbitol may result from stimulation of gastric secretion of intrinsic factor. Greenberg *et al.* (59) suggest a connection between the high proportion of reducing sugars in intrinsic factor mucoprotein and the stimulatory effect of some carbohydrates on the uptake of vitamin B_{12}. Morgan & Yudkin (62) maintained rats in good health on diets devoid of thiamine but containing D-sorbitol; they attribute this to synthesis of thiamine in the gut. The results with vitamin B_{12} cannot be explained by intestinal synthesis alone, since increased absorption was directly demonstrated by means of radioisotopes, but there may be some mechanism common to both of these sorbitol effects.

Assay of vitamin B_{12}: its distribution in natural materials.—Most recent work on the assay of vitamin B_{12} has been concerned with the adaptation of standard procedures to special circumstances. Daisley (63) developed a sensitive method for the measurement of vitamin B_{12} in sea water with *Euglena gracilis* strain Z and used it to determine the variation with depth

of the vitamin B_{12} concentration in the sea (64). The same organism was used by Killander (65, 66) to determine vitamin B_{12} levels in human serum, and this proved more valuable than haematological examination in the diagnosis of vitamin B_{12} deficiency (67 to 71). A short account of these studies has been published (72). In the developing chick embryo, Fisher, Benson & Swenseid (73) reported an apparent synthesis of vitamin B_{12} but, as *L. leichmannii* was the assay organism, the higher values observed in the later stages of incubation may have been caused by unspecific growth factors. Ford (74) studied the uptake of vitamin B_{12} and its analogues by *O. malhamensis;* pseudovitamin B_{12}, factor A, and cyanocobalamin were taken up by the cells in roughly equal amounts, but the analogues competitively inhibited growth response to cyanocobalamin, apparently by blocking the "binding" mechanism.

FOLIC ACID AND RELATED COMPOUNDS

Biosynthesis of folic acid.—Present knowledge on the biosynthesis and breakdown of folic acid has been fully reviewed by Woods (75). He points out that the available evidence is based on work with a variety of different microorganisms and, since it would be unwise to assume a common biosynthetic pathway, the general picture of folic acid biogenesis is not yet clear. During the past year Wacker, Ebert & Kolm (76) have continued their studies on the utilization by *Enterococcus Stei* and other organisms of carboxyl-^{14}C-PAB. It was incorporated into a reduced folic acid compound, formylated in position 5 and containing at least three molecules of glutamic acid joined through a peptide linkage. The same compound was isolated from organisms grown with labelled PGA. In the presence of aminopterin, believed to block the formylation step, the compound was not formed and the activity was present in PGA, indicating that PGA was an intermediate in the synthesis of a 5-formyltetrahydropteroylpolyglutamic acid. *Enterococcus Stei* grown in a medium containing carboxyl-^{14}C-*p*-aminosalicylic acid produced a substance that stimulated the growth of *Leuconostoc citrovorum* but not *Streptococcus faecalis* (77). Its properties were similar to those of the compound described in the previous paper (76), but it had an additional hydroxyl group on the benzene ring. By means of enzyme studies, Katunuma, Shoda & Noda (78) traced the synthesis of folic acid by *Mycobacterium avium*. It takes place in two steps, the first being a coupling of L-glutamate with PAB which requires ATP, CoA, and Mg^{++} as cofactors. The second step appeared to be the coupling of xanthopterin with PABG, but in a later communication (79) the reaction was stated to be the synthesis of 2-amino-4-hydroxypteridine-6-carboxylic acid with PABG in the presence of ATP. Xanthopterin could be used only if cocarboxylase or biotin or both were also present. The possibility of a pteridine intermediate in the synthesis of PGA was demonstrated by Shiota (80), working with crude extracts of *Lactobacillus arabinosus*. In a system consisting of the crude extract, PAB or PABG, ATP, and MgCl$_2$, folic acid-active com-

pounds were synthesized. Dialysis of the extract resulted in a loss of synthetic ability which was partially restored by addition of 2-amino-4-hydroxymethylpteridine or the 6-carboxyaldehyde derivative. Considerably greater synthetic activity resulted if these compounds were first chemically reduced.

There have been a number of studies on the reduction of PGA by way of dihydrofolic acid to the tetrahydro form. Wright, Anderson & Hermans (81) described an enzyme preparation from *Clostridium sticklandii* that formed $PGAH_2$ and its diglutamate from PGA and pteroyldiglutamic acid (teropterin) respectively. It did not form tetrahydro derivatives under the conditions studied. Further purification of the enzyme (82) established the following reaction:

$$\text{teropterin} + \text{pyruvate} + \text{CoASH} \xrightarrow[\text{reductase}]{\text{pteridine}} \text{reduced teropterin } H_2 + \text{AcSCoA} + CO_2$$
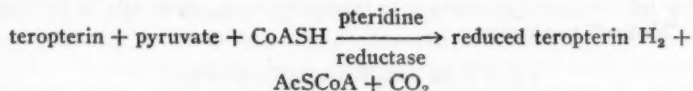
The enzyme was active also for mono- and triglutamyl pteridines and their N-10-formyl derivatives and was not inhibited by 4-aminopteroylglutamic acid. An enzyme system from chicken liver that reduces PGA and $PGAH_2$ to $PGAH_4$ has been prepared by Futterman (83) and by Zakrzewski & Nichol (84). Futterman's preparation appeared to consist of two enzymes, since TPNH was required for the reduction of PGA, whereas $PGAH_2$ was reduced by DPNH and by TPNH. 4-Aminopteroylglutamic acid blocked the utilization of the reduced pyridine nucleotides in both steps of the reduction. Osborn & Huennekens (85), by fractionation of chicken liver extracts, prepared dihydrofolic reductase which carries out the second step of the reduction sequence, as follows:

$$PGAH_2 + TPNH + H^+ \rightarrow PGAH_4 + TPN^+$$

The enzyme was inhibited noncompetitively by low levels of 4-aminopteroylglutamic acid or 4-aminomethylpteroylglutamic acid (86).

Metabolic role of folic acid and related compounds.—The function of tetrahydrofolic acid as a carrier of 1-carbon groups is well established. The metabolic reactions involving "active" formate and formaldehyde have been reviewed by Huennekens, Osborn & Whiteley (87). Greenberg & Jaenicke (88) have discussed the role of folic acid compounds in biosynthesis of purine nucleotides. The mechanism of formate activation has been studied by Whiteley, Osborn & Huennekens (89) with an enzyme from *Micrococcus aerogenes*. Their results suggest that phosphorylation of $PGAH_4$ is the first step, and the same enzyme catalyzes formylation of this postulated intermediate giving N-10-formyl-$PGAH_4$.

The synthesis of "active hydroxymethyl" by means of the formaldehyde-activating enzyme from pigeon liver has been achieved by Osborn, Vercamer, Talbert & Huennekens (90). The end product was identified by

enzymatic assays. In the same laboratory (91) a deacylase was detected in beef liver which effected the reaction: N-10-formyl—PGAH$_4$ + H$_2$O → HCOOH + PGAH$_4$. The deacylase probably serves to regenerate PGAH$_4$ in the absence of an acceptor for "active formyl." The role of folic acid, and its relationship to vitamin B$_{12}$ (see above), in the synthesis of methionine methyl has been studied in microorganisms and animal tissues. In an extract of an *E. coli* mutant requiring methionine or vitamin B$_{12}$ for growth, Helleiner, Kisliuk & Woods (92) showed that an equimolar mixture of formaldehyde and PGAH$_4$ could act as 1-carbon donor in the synthesis of methionine from homocysteine. Cyanocobalamin, glucose, ATP, and DPN were required for optimal synthesis. A different mutant, requiring glycine or serine for growth, could utilize PGAH$_4$ as carrier of 1-carbon units only if the organism had been grown in a medium containing cyanocobalamin (93). Dialyzed extracts of the organism behaved similarly, which suggests that a nondialyzable derivative of cyanocobalamin is necessary for the transfer by PGAH$_4$ of 1-carbon units to the methyl group of methionine. In an extensive study of the synthesis of methionine methyl, Nakao & Greenberg (94) demonstrated that the incorporation of isotope from 3-^{14}C-serine and ^{14}C-formaldehyde by an enzyme prepared from sheep liver required PGAH$_4$ or leucovorin, ATP, Mg^{++}, and DPN or TPN. Addition of vitamin B$_{12}$ to this system did not affect the reaction; however, if a cyanocobalamin complex such as that postulated by Kisliuk & Woods (93) in their microbial systems were necessary, it is likely that an enzyme preparation from animal tissues might already contain it.

In experiments with rats, Rabinowitz & Tabor (95) showed that in folic acid deficiency urinary excretion of formic acid, as well as formiminoglutamic was increased. Formiminoglutamic acid excretion was further increased by administration of histidine, as expected, but formic acid excretion was not; hence the formic acid did not arise either from histidine or from formiminoglutamic acid. It is suggested that formic acid, arising in the course of metabolic reactions not involving folic acid, is poorly metabolized in its absence. The metabolism of histidine in folic acid deficiency has been investigated by Baldridge (96). Liver levels of urocanase but not of histidase were lower in rats deprived of folic acid; this suggests that a folic acid derivative may be involved in the initial steps of the degradation of urocanic acid.

ASCORBIC ACID

Biosynthesis.—Mapson (97) has reviewed the biosynthesis of ascorbic acid in plants and animals.

The biosynthetic route from D-glucose to L-ascorbic acid in animals is well established. The essential steps are the conversion of D-glucose to D-glucuronic acid (or lactone), which is in turn converted to L-gulonic acid (or lactone) and thence to L-ascorbic acid. Thus, as a result of these interconversions, C-1 of glucose becomes C-6 of ascorbic acid.

The work has continued on the preparation of enzyme systems capable of catalyzing these changes. Isherwood *et al.* [unpublished data cited by Mapson (97)] have carried out experiments similar to those of Burns & Evans (98) with essentially the same results. They fractionated homogenates of rat liver and showed that the mitochondrial-microsomal fractions alone were responsible for the conversion of L-gulono- or L-galactono-γ-lactones into L-ascorbic acid; the free acids were not oxidized. The conversion of D-glucono-γ-lactone and of esters of D-galacturonic acid to ascorbic acid could not be accomplished by the particulate fractions alone; the whole homogenate was necessary. However, Chatterjee, Ghosh, Ghosh & Guha (99) have reported that the microsomal fraction of goat liver contains a cyanide-requiring enzyme that catalyzes the conversion of D-glucurono-γ-lactone to ascorbic acid. Cyanide was not required for the conversion of L-gulonolactone to ascorbic acid.

Bublitz, Grollman & Lehninger (100) have given further evidence for the presence in rat liver and pig kidney of enzymes carrying out the following reactions:

(a) D-glucuronate + TPNH + H$^+$ $\rightleftharpoons$ TPN$^+$ + L-gulonate
(b) L-gulonate + DPN$^+$ $\rightleftharpoons$ (3-keto-L-gulonate) + DPNH + H$^+$
(c) (3-keto-L-gulonate) $\rightarrow$ L-ascorbate + H$_2$O
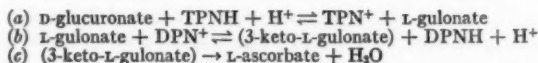
This scheme differs from those of Isherwood *et al.* (97) and Burns & Evans (98) in that the free acids rather than the γ-lactones are the immediate substrates and in that the oxidation of L-gulonic acid proceeds in the soluble fraction of the cell, though the final step of lactonization and isomerization requires the particulate fraction.

McCay, Carpenter & Caputto (101) made the interesting observation that the reduced synthesis of ascorbic acid by liver extracts from vitamin E-deficient rats could be restored by 2×10^{-3} MMnSO$_4$ to that by liver extracts from control rats. The addition of 4×10^{-3} MMgSO$_4$ was without effect.

Progress with the elucidation of the mechanism of ascorbic acid biosynthesis has allowed investigation of the step that limits synthesis in those species requiring a dietary supply of the vitamin, i.e., primates, including man, and guinea pigs. The report by Grollmann & Lehninger (102) that these species lack the enzyme for the conversion of L-gulonic acid to ascorbic acid has been followed by the demonstration by Burns (103) that whereas rat liver converts L-gulono-γ-lactone-1-^{14}C to L-ascorbic acid, liver from man and monkey does not. [Similar findings with guinea pig liver were reported by Burns *et al.* (104)].

Further studies are reported of the enzyme systems concerned with the interconversions in plants of D-galactose $\rightarrow$ D-galacturono-γ-lactone $\rightarrow$ L-galactono-γ-lactone $\rightarrow$ L-ascorbic acid. Thus Mapson & Isherwood (105)

demonstrated that an enzyme present in the nonparticulate fraction of homogenates of pea seedlings catalyzed a reaction between TPNH and esters of D-galacturonic acid to yield L-galactono-γ-lactone; the γ-lactones of D-glucuronic and D-mannuronic acids were reduced more slowly. L-Galactono-γ-lactone was oxidized to ascorbic acid by an enzyme present in the particulate fraction. This enzyme, L-galactono-γ-lactone dehydrogenase, was solubilized from the mitochondria of cauliflower florets by Mapson & Breslow (106), purified, and some of its properties described. Phenazine or cytochrome-c serves as electron acceptors for the enzyme which is a flavoprotein and which requires thiol groups for its activity. It shows a high specificity for its substrate and fails to catalyze the oxidation of D-mannono-, D-glucono-, D-galactono-, D-gulono, or L-gulono lactones.

However, the importance of such a route to ascorbic acid in plants has been strongly challenged by Loewus and his collaborators (107 to 110), who have an increasing body of radiochemical evidence that ascorbic acid is formed in the ripening strawberry from glucose by direct conversion, without inversion of the whole molecule, i.e., C-1 of glucose becomes C-1 of ascorbic acid. Detailed studies of the labelling patterns of ascorbic acid and of the free carbohydrates of strawberries stem-fed or injected with D-glucose-1-^{14}C, D-glucose-2-^{14}C, D-galactose-1-^{14}C, D-glucuronate-6-^{14}C; D-glucurono-lactone-6-^{14}C, L-arabinose-1-^{14}C or D-xylose-1-^{14}C provide strong support for the view that ascorbic acid is derived from a metabolic pool of carbohydrate probably identical with glucose-6-phosphate. Loewus, Jang & Seegmiller (110) summarized their findings in the following scheme:


```
triosephosphate _ _ _ _ _ pentose phosphate
   metabolism  ⇐ = = = =     metabolism
      ↕↑                        ↕↑
      ↕↓                        ↕↓
D-glucose ⇌ D-glucose-6-phosphate ⇌ 6-phospho-D-gluconic - - → L-ascorbic acid
                  ↕↑                    acid (lactone)
                  ↕↓
D-galactose ⇌ ⇌ D-glucose-1 phosphate ⇌ ⇌ D-glucuronic acid - - - - → D-galacturonic acid
```
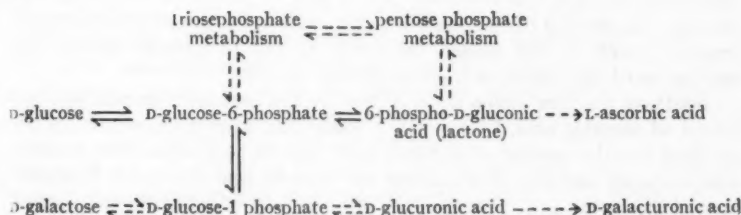
Ascorbic acid in metabolism.—The role of ascorbic acid in the synthesis of hydroxyproline, and thence in the biosynthesis of collagen, has been further studied by Gould (111, 112) who found that hydroxyproline formation in subcutaneously implanted polyvinyl sponges is analogous to its formation in granulation tissue. A direct, specific effect for ascorbic acid was demonstrated *in vivo* by the rapid hydroxyproline synthesis caused by the introduction of relatively small doses of sodium L-ascorbate into implanted sponges in scorbutic guinea pigs. Ascorbic acid could be replaced by dehydroascorbic acid, but glucoascorbic acid, isoascorbic acid and dihy-

droxymaleic acid were inactive. Gould stresses, however, that there may be alternative pathways for the formation of collagen since its formation in tissue culture was independent of ascorbic acid (113).

Kersten, Kersten & Staudinger (114) isolated from adrenal microsomes an enzyme system that catalyzed the oxidation of DPNH in the presence of ascorbic acid and molecular oxygen. The system required sulphydryl groups, and its absorption spectrum indicated that flavine and cytochrome-b_5 were components. They suggested that the hypothetical monodehydroascorbic acid acts as an intermediary electron acceptor. Ascorbic acid could not be replaced by dehydroascorbic acid, glutathione, or adrenaline, but D-isoascorbic acid had slight activity.

The disturbance of carbohydrate metabolism in scorbutic guinea pigs is discussed by Banerjee, Biswas & Singh (115), who found that their lowered glucose tolerance and decreased levels of liver and muscle glycogen were substantially improved by injections of insulin. The tissue concentration of α-keto acids was the same in normal and scorbutic animals and in animals injected with insulin, whereas the content of citric, malic, and lactic acids was higher in scorbutic than in normal guinea pigs and was lowered by injection of insulin. Thus these authors conclude that a deficiency of insulin is a major cause of abnormal carbohydrate metabolism in scurvy, rather than that abnormal metabolism is a direct effect of the lack of ascorbic acid on enzyme systems concerned with the oxidation of intermediates through the Krebs cycle. In a subsequent paper Banerjee & Singh (116) reported the effect of prolonged treatment with insulin on cholesterol metabolism in scorbutic guinea pigs. They found that the total content of body cholesterol increased in scorbutic guinea pigs as compared with controls; the increase resulted mainly from a higher level in the intestines. Prolonged treatment with insulin caused the levels to fall to normal values. The mechanism of these changes is not at present apparent.

Studying the free amino acids of the skeletal muscle of guinea pigs deprived of ascorbic acid, Ginter (117) found that after three weeks on the deficient diet the content of glutamic acid, leucine, and valine with methionine increased and that of glutamine and aspartic acid decreased. Comparisons of the amino acid content of the blood plasma of scorbutic and normal guinea pigs by Rangneker & Dugal (118) showed that in the former, the content of most acids fell. However the levels of phenylalanine, lysine, and histidine rose; the high level of phenylalanine was clearly related to the specific requirement for ascorbic acid for its metabolism (119).

A preliminary report by Souders & Varozza (120) suggests that the sex hormones may play a part in the regulation of blood, tissue, and urinary levels of ascorbic acid. Castrated male rats had lower levels in blood, liver, kidneys, and urine, though not in adrenals, than entire rats. The levels were increased by injection of testosterone. Injection of oestrone caused a further lowering of the blood level in castrated male rats; in castrated females it caused an increase, but with no effect on tissue levels.

The disappearance of ascorbic acid from the adrenal glands as a result of the stimulation of the secretion of adrenocortical hormones has been further investigated by studying the fate of the vitamin in the adrenal glands of hypophysectomized rats injected with ACTH. Slusher & Roberts (121) found that the ascorbic acid lost from the adrenal gland could be quantitatively recovered from the adrenal vein and that this loss preceded the secretion of corticosteroid by the gland. Using ascorbic acid-1-^{14}C, Salomon (122, 123, 124), showed that ascorbic acid exists in the adrenal gland in "free" and "bound" forms. The injection of ACTH caused the release into the adrenal vein of ascorbic acid from the bound form. The ascorbic acid recovered from the vein was not in the reduced form which suggested its participation in a chemical reaction before release. The "free" form of ascorbic acid in the adrenal was in equilibrium with ascorbic acid in the blood and was unaffected by ACTH.

Catabolism.—It is well established that rats and guinea pigs metabolize the carbon chain of ascorbic acid and that the main excretory products are CO_2 and oxalic acid. There is now evidence that the pathway of this breakdown in the rat differs from that in the guinea pig. Thus Chan, Becker & King (125) studied the metabolism of L-ascorbic acid-1-^{14}C in preparations of guinea pig liver and showed that the first step in the breakdown was conversion to dehydroascorbic acid which was further degraded to yield L-xylose, CO_2, and oxalate; whereas Burns, Kanfer & Dayton (126) found that preparations from rat kidney also oxidized ascorbic acid to the dehydro form, but the subsequent decarboxylation yielded no detectable amount of L-xylose, L-xylulose, or L-xylosone.

The metabolism of L-ascorbic acid in man presents a quite different picture. Hellman & Burns (127) have now published full details of their earlier findings (128) with L-ascorbic acid-1-^{14}C. Essentially all the administered ^{14}C was excreted in the urine as L-ascorbic acid, diketo-L-gulonic acid, and oxalic acid. Moreover, no ^{14}C was detected in respiratory CO_2. Hellman & Burns suggest that the inability of man to decarboxylate L-ascorbic acid may account for the slow rate of metabolism of this vitamin in man, in whom it has a half life of 16 days, compared with half lives of four days and three days in guinea pigs and rats, respectively. This difference may explain why man requires a much longer period of time on a vitamin C-free diet to develop scurvy than does the guinea pig.

The metabolic rates in rats and guinea pigs of D- and L-ascorbic-1-^{14}C acids were compared by Dayton & Burns (129), who found that in the rat the D- and L- isomers are both oxidized to CO_2 at about the same rate but that a much larger part of the dose of the D- isomer was excreted in the urine intact. In the guinea pig the D-isomer was oxidized to CO_2 and excreted much more rapidly than the L-isomer. In fact most of the D-ascorbic acid disappeared from the body in 24 hr. This finding prompted Dayton & Burns to reassess the biological activity of D-ascorbic acid when given to scorbutic guinea pigs at a high enough level to maintain tissue concentra-

tions comparable with normal levels of L-ascorbic acid. A preliminary experiment suggests that under such conditions the D-isomer does have vitamin C activity.

BIOTIN

There is still no knowledge of a clear-cut mode of action of biotin and relatively little attention is being given to its role in metabolism.

Further evidence for a function of biotin in CO_2 fixation has been reported. Bettex-Galland (130) showed that much less ^{14}C from $NaH^{14}CO_3$ was incorporated into aspartic acid by livers from chicks deficient in biotin than by livers from normal chicks. Lichstein (131) has given further data demonstrating the occurrence of bound biotin in purified oxalacetic carboxylase from chick livers. The purest preparation contained 3 mμg. of biotin per mg. of protein. Woessner, Bachhawat & Coon (132) measured the activities in the livers of biotin-deficient chicks of the two enzymes required for the carboxylation of β-hydroxyisovaleryl CoA and found that the activity of the CO_2-activating enzyme was unchanged, whereas β-hydroxyisovaleryl CoA carboxylase was completely lacking. It appeared, therefore, that biotin was concerned, not with CO_2 fixation, but with carboxyl transfer; the significance of the finding in relation to the mode of action of biotin is difficult to assess since all attempts to restore carboxylase activity in the deficient liver were unsuccessful.

A possible role for biotin in fatty acid metabolism is supported by results of Rossi, Rossi & Rossi (133, 134), who found that biotin deficiency impaired the formation of acyl CoA in rat livers, and of Wakil, Titchener & Gibson (135) who showed that an enzyme fraction from avian liver, which catalyzed the synthesis of fatty acids with CoA, contained 200 to 250 mμg. of biotin per mg. protein; the system was inhibited by avidin, and the inhibition was reversed by biotin. Gram & Okey (136) found that the preferred pathways of utilization of acetate-2-^{14}C by biotin-deficient rats were oxidation to CO_2 and synthesis of glycogen, whereas in nondepleted pair-weighted controls acetate was preferentially utilized for the synthesis of liver lipide and cholesterol.

Lichstein & Ferguson (137) made the interesting observation that the transport of biotin into cells of L. arabinosus requires energy and that it is inhibited by homobiotin, a biotin homologue.

A biotin-requiring mutant strain of E. coli and a strain of Propionibacterium pentosaceum proved to be unsatisfactory assay organisms (138). Dhyse & Hertz (139) found that the giving to rats of actithiazic acid

$$
\begin{array}{c}
\text{CO} \longrightarrow \text{NH} \\
| \qquad\quad | \\
\text{CH}_2 \quad \text{CH(CH}_2)_4\text{CH}_2\text{COOH} \\
\diagdown \; \diagup \\
\text{S}
\end{array}
$$
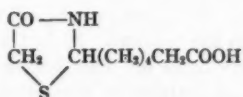
caused a large increase in the excretion in the urine and faeces of an "avidin-uncombinable" form of biotin. This biotin vitamer was also found in the supernatant of *E. coli* cells and its concentration was increased 5 to 10 times when the cells were grown in the presence of actithiazic acid.

VITAMIN B₆

General.—Christensen (140) made a detailed study of the structure of the Schiff bases resulting from the interaction of amino acids, peptides, and proteins with pyridoxal and pyridoxal-5′-phosphate (Figure 1). From measurements of absorption spectra, infrared spectra, and pK values, he suggests that pyridoxal or pyridoxal phosphate (I) reacts with an amino group to form first a hydrogen-bonded yellow imine (II) which then loses either the chelated proton or the proton from the pyridine N to form either III or IV; III may then hydrate to the carbinolamine V, and IV do likewise to form VI.

In studies of nonenzymatic reactions catalyzed by vitamin B₆, Bergel, Bray & Harrap (141) showed that vanadium was considerably more effective than Al³⁺ as an activator of pyridoxal phophate in the decomposition of cysteine. This desulphydraselike reaction took place at room temperature and had an optimum pH of six. Ichihara *et al.* (142) found that a tryptophanaselike reaction was effected by heating an alkaline solution of tryptophan with pyridoxal and Cu⁺⁺ at 100°. Pyridoxine, pyridoxamine, or salicylic acid could not substitute for pyridoxal, but salicylaldehyde showed some activity.

An interesting application of the ability of α-amino acids to undergo transamination with pyridoxal was developed by Kalyankar & Snell (143) for the differentiation of α-amino acids from β-amino acids and other primary amines. After chromatographic separation of the amino compounds, the paper was sprayed with pyridoxal hydrochloride and heated to effect transamination; subsequent spraying with ninhydrin revealed the pyridoxamine spots as orange zones.

Korte & Bannuscher (144) reported that homogenates of pig liver converted pyridoxal phosphate to 4-pyridoxic acid in good yield; other vitamin B₆ compounds were not converted. Pogell (145) has published further details of an oxidative system from rat liver which catalyzes the oxidation of pyridoxamine or pyridoxamine phosphate to pyridoxal or pyridoxal phosphate according to the equation: $PAMP + \frac{1}{2} O_2 \rightarrow PALP + NH_3$. The enzyme is different from known amine oxidases; after partial resolution from its prosthetic group it was reactivated by FAD or riboflavin-5′-phosphate.

Methods.—A fluorimetric method for the differential assay of pyridoxal and pyridoxamine at concentrations of about 0.1 μg. per ml. was developed by Coursin & Brown (146). The method depends on the measurement of fluorescence at 400 mμ before and after destruction of pyridoxal with 30 per cent hydrogen peroxide and of pyridoxamine with ultraviolet light.
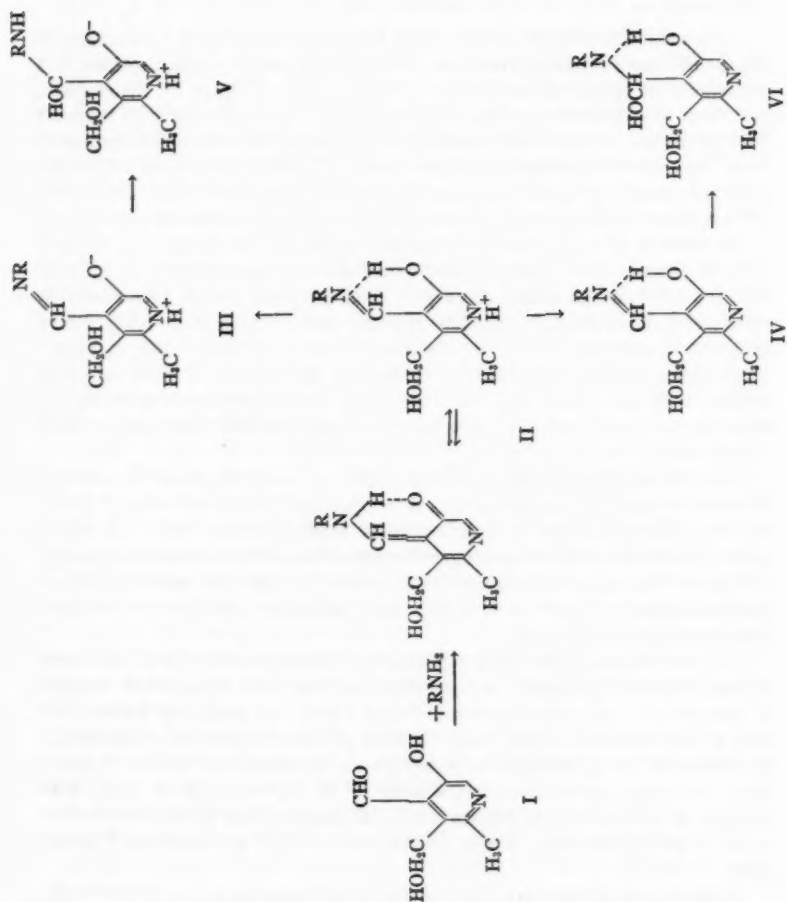
FIG. 1. Pyridoxal and Schiff base Structures.

Boxer, Pruss & Goodhart (147) have modified and increased the sensitivity of the manometric method of Umbreit, Bellamy & Gunsalus (148) and have used it to measure pyridoxal phosphate in whole blood and leucocytes of several animal species. The method proved insufficiently sensitive to detect pyridoxal phopshate in 1 ml. samples of the blood of about 80 per cent of human adults whose blood must have contained, therefore, less than 10 mμg. per ml. However, the method allowed the measurement of the pyridoxal content of leucocytes from 5 to 10 ml. of human blood. Wachstein *et al.* (149, 150) have used Boxer's method to measure pyridoxal phosphate in the leucocytes of pregnant women (149), and in the blood and tissues of rats with developing vitamin B$_6$ deficiency (150). An alternative method based on the coenzyme properties of pyridoxal phosphate was developed by Wada, Morisue, Sakamoto & Ichihara (151). It depends on the colorimetric measurement of indole formed from tryptophan when pyridoxal phosphate is added to apotryptophanase from *E. coli;* as little as 0.03 μg. of pyridoxal phosphate could be estimated. Pyridoxal, pyridoxine, and pyridoxamine are not active.

Antivitamins and antagonists.—One of the proposed mechanisms for the action of isonicotinic acid hydrazide has been the inhibition of enzyme systems requiring pyridoxal phosphate. Youatt (152) considered that it was unlikely that the inhibition of an enzyme system present in many bacteria could account for the highly specific activity of INH in low concentrations against species of *Mycobacterium* and showed that transaminase activities in sensitive and resistant strains of *Mycobacterium tuberculosis* were equally affected by INH and that transaminase activity was found in extracts of cells which had been exposed to INH under bactericidal conditions. Furthermore, she found that stable hydrazones of INH, including pyridoxalisonicotinyl hydrazone, had tuberculostatic activity similar to that of INH and did not inhibit transaminase activity. She concluded that it is improbable that the bactericidal activity of INH can be explained by its action on a transaminase or other enzyme requiring pyridoxal phosphate.

Daily doses of 300 to 900 mg. of isonicotinic acid hydrazide given in the treatment of tuberculosis caused an increased excretion of kynurenine and xanthurenic acid in tryptophan load tests carried out by Price, Brown & Larson (153), though not in those conducted by Sass & Murphy (154). However, Sass & Murphy found lower levels of glutamic-oxalacetic transaminase in the blood of patients receiving the drug.

The antivitamin B$_6$ activity of toxopyrimidine (2-methyl-4-amino-5-hydroxy methyl pyrimidine) in rats, mice, and bacteria has been further investigated. Hiroaka (155) showed that its inhibitory effects on the growth of several strains of lactic acid bacteria was reversed more readily by pyridoxal or pyridoxamine than by pyridoxine. Nishizawa, Kodama & Kooka (156) found that the vitamin B$_6$ level in the tissues was lowered in rats receiving toxopyrimidine and that the liver damage resulting from it was

more extensive than that occurring in vitamin B_6 deficiency. Two analogues of toxopyrimidine, 2,5-dimethyl-4-amino-pyrimidine and 2,6-dimethyl-4-aminopyrimidine, were also effective antagonists to vitamin B_6 in rats (157). The quantitative interrelationships for antagonism to vitamin B_6 in mice of toxopyrimidine and several of its analogues was studied by Nishizawa, Kodama & Miyake (158) who found that injected doses of 0.25 mg./gm. body weight were counteracted by a simultaneous injection of one fiftieth of the amount of pyridoxine, whereas doses of 1.5 mg./gm. required 1 mg./gm. of pyridoxine.

The cause of the convulsions that occur after injection of toxopyrimidine is discussed by Nishizawa (159), who found that, owing to lower levels of glutamic acid decarboxylase, γ-aminobutyric acid levels in the brain were lowered just prior to the onset of convulsions. Attempts to suppress convulsions by injecting γ-aminobutyric acid were unsuccessful, possibly because it was not transferred to the brain. Injections of β-hydroxy-γ-aminobutyric acid did suppress the convulsions caused by toxopyrimidine and by certain other convulsive agents. Likewise, Holtz & Westermann (160) suggest that the hallucination-producing action of adrenaline, adrenalochrome, and similar compounds results from interference with glutamic acid metabolism in the brain through inhibition of glutamic acid decarboxylase.

Another antitubercular drug, cycloserine (D-4-amino-3-isoxazolidinone), is also believed to interfere with vitamin B_6 enzyme systems. Yamada, Sawaki & Hayami (161) and Aoki (162) have shown that it combines with pyridoxal in bacteria to form a complex. It markedly inhibits transaminases from several strains of bacteria (163) and it inhibits the nonenzymic formation of indole from tryptophan by pyridoxal and Cu^{++} (162).

Vitamin B_6 enzymes.—Further work has confirmed the earlier report (164) that muscle phosphorylase contains pyridoxal phosphate (165, 166). Studies of the spectral properties of phosphorylase by Kent, Krebs & Fischer (167) and of its reaction with sodium borohydride by Fischer, Kent, Snyder & Krebs (168) indicate that pyridoxal phosphate is bound to the enzyme as a substituted aldamine (I) which is converted to a Schiff base form (II)

I   II
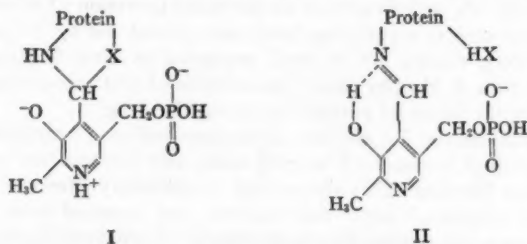
and eventually split off from the enzyme by treatment with acid, base, or urea. Reduction with sodium borohydride gave a pyridoxylamine derivative

from which pyridoxal could not be liberated. The reduced enzyme was degraded with chymotrypsin to yield ε-N-pyridyllysine. Furthermore, and most interestingly, the reduced enzyme was fully active in the phosphorylase reaction.

Eggleston (169) has provided further evidence for the general mechanism proposed by Metzler *et al.* (170) for reactions catalyzed by pyridoxal phosphate in which there is a requirement for a metal ion. He found that the activity of seven decarboxylases from *E. coli* and *Clostridium welchii* was increased up to 45 per cent by bivalent ions; Mg^{++} was usually the most effective. Histidine decarboxylase from *E. coli* was an exception in that, although it was stimulated by pyridoxal phosphate, the cations tested had no effect.

Pyridoxal phosphate was shown by Matsuo & Greenberg to be the coenzyme of homoserine deaminase-cystathionase from rat liver (171) and to be the probable coenzyme of histidine carboxylase (172), γ-aminobutyric-α-ketoglutamic transaminase (173) and diaminopimelic acid decarboxylase (174). The enzyme systems responsible for the synthesis of δ-aminolevulinic acid in chick erythrocytes (175, 176) and in cells of *Rhodopseudomonas spheroides* (177, 178) require pyridoxal phosphate as one cofactor.

The possible role of pyridoxal phosphate in the incorporation of carbon from formaldehyde-^{14}C or of C-3 from serine-3-^{14}C into the methyl group of methionine was studied with inconclusive results by Nakao & Greenberg (179) and by Doctor (180).

Cysteine sulphydrase was purified by Yamada & Tokuyama (181) from a gram-negative soil organism and partially purified from rat liver. Ichihara *et al.* (142) studied the properties of tyrosinase and tryptophanase from *E. coli phenologenes;* the dialyzed enzymes required pyridoxal phosphate, K^+ and NH_4^+ for activity and were inhibited by metal chelating agents and by —SH inhibitors.

MISCELLANEOUS

Mevalonic acid (β-methyl-βδ-dihydroxyvaleric acid).—Factors influencing incorporation of mevalonic acid into cholesterol by rat liver homogenates have been studied by Wright & Cleland (182). In the same system, mevaldic acid appeared to be a precursor of mevalonic acid (183). The direct incorporation of mevalonic acid into squalene was demonstrated with yeast extracts (184) and rat liver homogenates (185). The synthesis of rubber from mevalonic acid by means of a crude enzyme in the latex of *Hevea brasiliensis* has been achieved by Park & Bonner (186).

Carnitine (γ-trimethylamino-β-hydroxybutyric acid).—The history, chemistry, and biological significance of carnitine have been extensively reviewed by Fraenkel & Friedman (187). A chemical method has been devised by Friedman (188) whereby it can be assayed in the presence of choline and betaine, and in crude animal tissues. Carnitine was shown by Lecoq (189) to bring about a diminution in plasma alkali reserve in rabbits,

whereas Goetsch's vitamin T (190) did not have this effect. A papain hydrolysate of casein, essential for the development of the larvae of the rice meal beetle, could only be partly replaced by carnitine (191).

Unidentified growth factors.—The position regarding unidentified growth factors for poultry remains confused. Many papers indicate the existence of several such factors, some of them mineral. A useful review of the literature by Wakelam & Jaffé (192) accompanies their report of a stimulation of growth of chicks and poults by malt distillers' solubles. The activity which remained after ashing was traced to Mn (193), in contrast to earlier work of the Texas group (194) who found Mo responsible for the growth-stimulating effects of their sample of distillers' solubles. The possibility that the growth-promoting factor in fish meal may be peptide in nature has been postulated (195, 196), since its activity disappeared on acid hydrolysis but was retained after enzyme digestion.

Wiesner & Yudkin (197) continued their studies in rats of a nutritional substance in liver (Factor R) necessary for the production of viable young.

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NUTRITION[1,2]

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Recent previous reviews of this topic (1, 2) have dealt with protein and calcium nutrition, amino acid interrelationships, energy exchange, obesity, and atherosclerosis. In view of the intense research activity and great interest in studies of the effect of dietary fats upon cellular physiology, lipide metabolism, and health generally during the past two years, this review will deal with that subject. Specific topics which will be covered are (*a*) essential fatty acids, (*b*) nutritional aspects of experimental and clinical atherosclerosis and (*c*) the function of the fat-soluble vitamins. It is hoped that the reviewer's own interest and preoccupation with certain aspects of this field have not unduly disturbed his sense of perspective.

ESSENTIAL FATTY ACIDS

The importance of certain polyunsaturated fatty acids in the diet of the rat was first demonstrated by Burr & Burr (3). From their experiments and subsequent ones, the deficiency syndrome in this species was shown to consist of growth failure, scaliness of the skin, necrosis of the tail, cardio- and hepatomegaly, testicular tubular degeneration, hematuria, reproductive failure, polydipsia, and hypermetabolism. All of these abnormalities could be prevented or cured by the administration of 20 to 100 mg. of linoleic acid daily, females requiring less than males. Additional species, including mouse, guinea pig, pig, dog, rabbit, man, and even certain insects, have been shown to require linoleic acid, so this nutritional requirement seems to be very general (4, 5). In all of the mammals studied, the prime indicator of the deficiency state has been a dermatitis associated with some degree of growth failure. It has been virtually impossible to demonstrate the deficiency in adult organisms, presumably because of a reduced requirement as well as increased stores of linoleic and related acids in organ and depot fat. In a period when

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the term "essential fatty acid" (EFA) is being used with abandon, it might be well to ask the question, "Essential for what?" The Burrs (6) defined the essentiality of linoleic acid in terms of its ability to prevent or cure all of the signs of fatty acid deficiency in the young rat; this view was recently reaffirmed by Holman (5). Similar criteria should be applied to the definition of essentiality of linoleic and related fatty acids in other species, including man. Using the fat-deficient weanling rat as a bioassay organism, it has been established that linoleic (9,12-octadecadienoic acid), arachidonic (5,8,11,14-eicosatetraenoic acid), and γ-linolenic acid (6,9,12-octadecatrienoic acid) are highly potent in stimulating growth and curing dermal and other signs of EFA deficiency (7, 8). Ordinary α-linolenic acid (9,12,15-octadecatrienoic acid) and many highly unsaturated fatty acids found in fish oils of the "linolenate" type were found to stimulate growth but not to cure dermal signs. The chemical configuration which appears to be essential in the sense defined above is the $CH_3-(CH_2)_4-(CH=CH-CH_2)_2-R$ moiety. Counting paradoxically from the methyl end of the chain, this would represent a 6,9-diene. Additional unsaturation of the divinylmethane type or elongation of the chain toward the carboxyl end does not appear to change the quality of the organism's response but may alter its quantitative value. Arachidonic acid has been reported to be 3 to 5 times as potent as linoleic acid in curing EFA disease in rats (9), whereas homolinoleic acid (11,14-eicosadienoic acid) has only 40 per cent of the activity of linoleic acid (8). On the other hand, additional unsaturation of the divinylmethane type toward the methyl end, as in α-linolenic and related acids, destroys the essentiality of these fatty acids for EFA deficiency disease. In a strict sense, only linoleic acid is essential, since the mammalian organism can synthesize γ-linolenic and arachidonic acids from linoleic acid (*vide infra*). Unnatural isomers of the natural *cis, cis* linoleic acid possess no EFA activity. *Trans, trans* linoleate and *cis, trans* linoleate (7) are ineffective and, under certain conditions, may actually function as antimetabolites (10). The use of the word essential to described polyunsaturated fatty acids instrumental in causing hypocholesterolemia in man represents a misuse of the term on two counts: (*a*) essentiality of fatty acids was originally defined in terms of protection of the skin, (*b*) the type of unsaturation required for one effect is not identical with that required for the other.

Biosynthesis of polyunsaturated acids.—Although it has been known for some time on the basis of feeding experiments with linoleic and linolenic acids, that rats and chicks can convert linoleic acid to arachidonic acid and linolenic acid to a hexaenoic acid (4), the precise mechanism of these conversions has not been elucidated until recently.

The conversion of linoleic acid to arachidonic acid in the mammal has been studied with isotopic techniques by Klenk (11) in Germany and Mead and co-workers (12) in the United States. Mead, Steinberg & Howton (13) showed that acetate-1-C^{14} was rapidly incorporated into carcass arachidonic but not linoleic acid in weanling rats. Degradation of the labeled arachidonic acid revealed that all of the label was in the carboxyl group consistent with

C_2 addition to the carboxyl end of exogenous linoleic acid. Lipsky and associates (14) have found that acetate-1-C^{14} is also not incorporated into linoleic acid in man. On the basis of distribution of C^{14} among dicarboxylic acids derived from polyenoic acids from liver phosphatide in rats given C^{14}-acetate, Klenk (11) concluded that acetyl addition to both linoleic and linolenic acids was the basic mode of synthesis of the highly polyunsaturated acids of liver and brain phosphatide. In subsequent experiments, Steinberg *et al.* (15) found that, after administration of linoleate-1-C^{14} to rats, tagged arachidonic and docosapentaenoic acids were obtained. Degradation of arachidonate in this case showed essentially all of the activity in carbons one and three, consistent with oxidation of some of the linoleate to acetyl CoA (16) and reincorporation into arachidonate in the C_1 position. In further experiments to identify the intermediates in the biosynthesis of arachidonic from linoleic acid, Mead & Howton (17) studied the incorporation of γ-linolenate-1-C^{14} into arachidonic acid and found almost quantitative conversion into arachidonate-3-C^{14} adding further to the biological evidence (*vide supra*) that γ-linolenate is an intermediate in the conversion of linoleate to archidonate. Whether dehydrogenation or acetyl addition occurs first in this two-step reaction has not yet been decided. On the basis of the occurrences of homo-γ-linolenic acid (8,11,14-eicosatrienoic acid) in beef liver phosphatide (18), the latter seems likely, as shown in Figure 1.

$$CH_3-(CH_2)_4-(CH=CH-CH_2)_2-(CH_2)_6-COOH$$

α-Linoleic Acid

(9, 12-octadecadienoic acid)

$$CH_3-(CH_2)_4-(CH=CH-CH_2)_3-(CH_2)_3-COOH$$

γ-Linolenic Acid

(6, 9, 12-octadecatrienoic acid)

$$CH_3-(CH_2)_4-(CH=CH-CH_2)_3-(CH_2)_5-COOH$$

Homo-γ-linolenic Acid

(8, 11, 14-eicosatrienoic acid)

$$CH_3-(CH_2)_4-(CH=CH-CH_2)_4-(CH_2)_2-COOH$$

Arachidonic Acid

(5, 8, 11, 14-Eicosatetraenoic Acid)
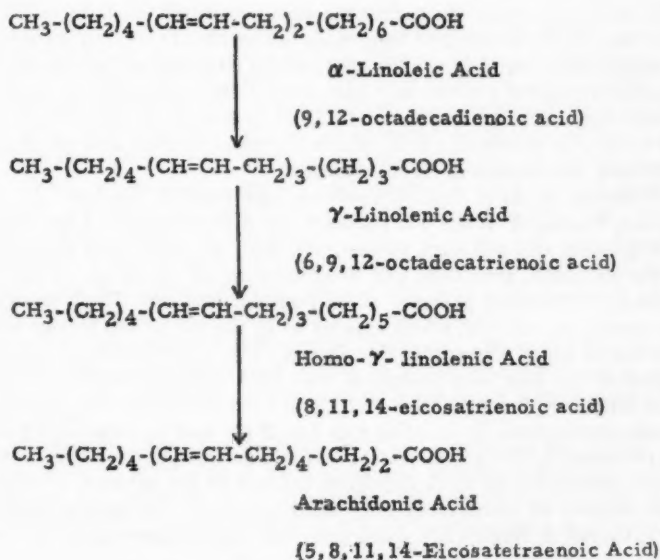
FIG. 1. Conversion of linoleate to arachidonate.

Witten & Holman (19) showed that rats deficient in EFA and pyridoxine synthesized less arachidonic acid when given linoleate than those deficient in EFA alone or the doubly deficient rats given pyridoxine. The precise role of pyridoxine is not clear because "B_6-type reactions" are not apparent in this conversion. Presumably the C_2 addition to linoleate involves a β-ketoacyl-thiolase catalyzed coupling of acetyl CoA and linolyl CoA followed by a TPNH-dependent reduction (20). The specific acyl CoA dehydrogenases concerned with 9 and subsequent divinylmethane dehydrogenations have not, however, been studied.

The series of reactions presented in Figure 1 appear to represent a sequence that is of general significance in the metabolism of C_{18}-fatty acids. Steinberg and associates (21) found that when linolenate-1-C^{14} was fed to rats, inappreciable amounts of linoleic and arachidonic acids were found. Instead the radioactivity resided in eicosapentaenoic, docosapentaenoic, and docosahexaenoic acids which were derived from linolenate by chain length extension of the carboxyl end accompanied by 1,4-diene formation from the existing unsaturation toward the carboxyl end. Klenk (18) has identified 4,7,10,13,16-docosapentaenoic acid from brain phosphatide and 7,10,13,16,19-docosapentaenoic acid from liver phosphatide which could be derived from linoleic and linolenic acids, respectively. The process, furthermore, appears to occur with fatty acids which can be synthesized *de novo* in the body, such as oleic acid. Mead & Slaton (22) found that 5,8,11-eicosatrienoic acid is the trienoic acid which accumulates in the lipides of EFA-deficient rats (23), and is apparently synthesized via analogous reactions. Saturated fatty acids are the first products of lipogenesis in animals (14, 24). In the absence of exogenous essential fatty acids required for intracellular lipoproteins, the organism appears to produce polyunsaturated fatty acids within the limits of its enzymatic capacity. In addition to 5,8,11-eicosatrienoic acid, Mead (25) has noted that palmitoleic acid, which resembles linoleic acid in physical properties, accumulates in EFA deficiency.

Pathology of EFA deficiency.—Ramalingaswami & Sinclair (26) and Hansen, Sinclair & Wiese (27) studied the histopathology of the skin in EFA-deficient rats and dogs, respectively. Both species showed hyperplasia of the epidermis, acanthosis and hyperkeratosis of the lining of the hair follicles, and keratotic plugging of the follicular openings. The histology of the stratum granulosum, which appears to be the site of the barrier to the diffusion of water, was appreciably altered (28). Degeneration of testicular epithelium has long been associated with EFA deficiency in the rat (29). Aaes-Jorgensen, Funch & Dam (30) have reported severe degeneration of spermatogenic tissue in weanling rats fed 28 per cent hydrogenated peanut oil, prevented by 100 mg. of methyl linoleate per rat per day. This is interpreted as evidence of EFA deficiency induced by the presence of various *trans* isomers of unsaturated fatty acids present in the hydrogenated oil (31). Carroll & Noble (32) noted decreased spermatogenesis in male rats

given a powdered meal plus 10 per cent erucic acid (13-docosenoic acid) and suggests that this unphysiological monoethenoid acid may block the utilization of the essential fatty acids. Hill *et al.* (33) noted aortic lesions of Mönckeberg type in young swine fed an EFA-deficient diet. The lesions were not true atheroma but involved the media rather than the intima and resembled the vascular lesions reported by Wilgram and co-workers (34, 35) in the choline-deficient rat.

The suggestion by Sinclair (36) that human atherosclerosis is caused by EFA deficiency does not seem to be supported by the facts. EFA deficiency in man, as in other species, has been observed only in the growing organism on a fat-free diet (37) in which dermatitis, inefficiency of calorie utilization, and inversion of the diene/triene fatty acid ratio in the serum are seen (38, 39, 40). Since the amount of linoleate required to prevent or cure this condition in the infant is less than 1 per cent of total calories, the requirement for prevention of the same syndrome in the adult, reasoning from animal experiments and studies on older children (41) should be even lower. Inasmuch as the intake of linoleic acid by an "average American" ingesting a diet containing 40 per cent of calories from fat is about 4 per cent of total calories (42, 43), it seems highly unlikely that EFA deficiency could account for the atherosclerosis seen in adults in the United States. Even in Capetown, South Africa, where the incidence of coronary disease varies widely among Bantu, Cape colored, and white groups, no dietary deficiency of linoleic acid is evident from analysis of the fats eaten. The intake of linoleic acid in these groups (1.1, 1.8, and 1.9 per cent of total calories, respectively) correlated directly instead of inversely with serum cholesterol values and incidence of coronary artery diseases. James and associates (44) studied the fatty acid composition of the serum lipides of twelve patient with coronary artery disease and twelve "healthy" controls by gas phase chromatography. They found no evidence of deficiency of linoleic or arachidonic acids in the lipides of red cells, plasma phospholipides, or plasma neutral fat in these two groups. If anything, the controls had higher percentages of myristic and stearic and less oleic acid in the neutral fraction than did the patients with coronary artery disease. Caren & Carbo (45) also found no differences in the iodine numbers of the fatty acids from cholesterol esters, phospholipides, triglycerides and nonesterified fatty acids in serum of patients with coronary artery disease and normal controls.

Even if one assumes a hypothetical "imbalance" between saturated and essential fatty acids present in the diets of populations victimized by coronary disease, there is scant evidence that EFA deficiency is the basic etiology of atheroma.

Pathological physiology in EFA deficiency.—Although the precise biochemical events which lead to the microscopic and gross pathology in EFA deficiency are unknown, some biochemical changes associated with EFA deficiency have been reported. It is known, for example, that the skin and the

testes, two tissues vulnerable to EFA deficiency, are rich in arachidonic acid (46). Aaes-Jorgensen & Holman (47) have studied the changes in the polyenoic fatty acids of heart and testes in the rat a fat-free diet and have shown that the reciprocal decrease in diene and increase in triene (5,8,11-eicosatrienoic acid) is most quickly seen in the heart lipides. The next sensitive tissue was the testes with inversion of diene/triene ratio preceding the morphological changes by several weeks. Wiese, Hansen & Bowman (48) studied the effect of EFA deficiency in the dog upon the distribution of polyunsaturated fatty acids in the lipides of serum. The phospholipides contained the most arachidonic acid and the cholesterol esters the most linoleic acid, a finding also true of rat liver (49). In EFA-deficient dogs, the serum dienoic and tetraenoic acids decreased while trienoic acids increased.

Disturbances in cholesterol transport resulting in hypocholesterolemia associated with accumulation of liver cholesterol have been reported in EFA-deficient rats (50). Klein (51, 52) compared the polyunsaturated fatty acid content of liver and plasma cholesterol esters under various conditions of linoleate feeding and concluded (*a*) the concentration of cholesterol ester varied phasically with the amount of linoleate fed—high with none, low with some, and high with more; (*b*) the liver cholesterol esters tended to be more saturated than serum esters and reflected the dietary intake of polyunsaturated fat better, a finding corroborated by Mukherjee *et al.* (53) and Okey & Harris (49). Mukherjee & Alfin-Slater (54) found low rates of incorporation of acetate-1-C^{14} into liver cholesterol of animals on a fat-free diet as compared to controls fed 15 per cent cottonseed oil.

Peifer & Holman (55) reported that the addition of 1 per cent cholesterol to the diets of weanling rats fed an EFA-deficient diet accelerated the onset of the skin signs. Attempts to induce EFA deficiency in adult rats by feeding them cholesterol plus cholic acid led to marked hypercholesterolemia but not to a depletion of polyunsaturated fatty acids in testes or heart (56). The measurement of plasma trienoic acid in humans given alternately butterfat and corn oil as a source of dietary fat showed insignificant changes compared with those noted in EFA deficiency in dogs or after a prolonged fat-free diet in man (57). Antonis (58) found no differences in the per cent serum diene and triene in young adult Bantus and Europeans living in South Africa, despite sizeable differences in serum cholesterol. Geriatric patients with atherosclerosis had a lower percentage of diene but no increase in triene. The addition of safflower oil to both groups increased the diene without change in triene. The modification of serum lipides by feeding large amounts of dietary linoleic acid resulted in modification of milk fat in man (59) and rats (60) and egg fat in hens (61, 62).

The hypermetabolism of the EFA-deficient rat has been studied by Panos and co-workers (63, 64, 65) and Sinclair (28). The increased insensible loss of water was found insufficient to explain the increased metabolic rate. The possibility that uncoupling of oxidative phosphorylation causes the high

metabolic rate and the inefficiency of caloric utilization was tested directly by Klein & Johnson (66). In association with a loss of dienoic and tetraenoic acids and an increase in trienoic fatty acids in mitochondria, there was an alteration in uptake of inorganic P^{32} into organic fractions. Levin, Johnson & Albert (67) found an increased Qo_2 with pyruvate and a decreased P/O ratio in mitochondria from rats fed fat-free diets. Similar dissociation of oxidative phosphorylation could be induced in normal mitochondria by exposure to hypotonic solutions. These experiments suggest that the loss of polyunsaturated fatty acid from the mitochondrion may cause alterations in lipoprotein structure which may, in turn, result in biochemical alterations. The erythron of the EFA-deficient rat also seems to be more fragile (68).

ATHEROSCLEROSIS

Of the environmental factors contributing to the etiology of atherosclerosis, nutrition appears to be gaining prominence. It seems clear, however, that atherosclerosis is a disease of multiple causation representing a complex interaction between the organism and its total environment (69). Contrary to the more classical situation in which the agent of a disease arises from the environment such as in infection, allergy, drug addiction, or vitamin deficiency disease, in atherosclerosis the agent appears to arise within the host. Futhermore, "activity" of the agent depends upon many possible host-environment interactions. In such an ecologic framework, Olson (70) has suggested that the plasma β-lipoproteins might be considered the agent of atherosclerosis, realizing that agents of disease are necessary but not sufficient causes for illness. In humans, where fat transport is carried out at relatively high concentrations of β-lipoproteins such host factors as blood pressure and turbulence of flow, structure and metabolism of arterial tissue, and coagulability of the blood may be critical in the pathogenesis of the disease and the precipitation of clinical events.

Since the liver is largely responsible for the elaboration of the plasma β-lipoproteins, this organ might be considered the source of the agent. The extent to which environmental factors, including diet, influence the concentration of the agent will depend in large upon the extent to which they can alter the rates of synthesis, secretion, and catabolism of these lipoproteins. Environmental factors, of course, may modify other etiologic factors besides the agent, and thus influence the course or progression of the disease. Host factors, such as the basic enzymic differentiation of cells, endocrine secretions, and nervous factors, may influence both the concentration of agent and susceptibility of arterial tissue to alteration. In relating any environmental variable to a disease of multiple etiology, one must be aware of the influence of many other factors upon the final outcome. The working hypothesis that diet may influence liver function in such a way as to control, in part, the β-lipoprotein concentration in the plasma and hence regulate, in

part, atherogenesis and, in part, clinical events associated with atherosclerosis is diagrammed below (71) :

DIET→LIVER→PLASMA β-LIPOPROTEINS→ATHEROGENESIS→

CLINICAL EVENT
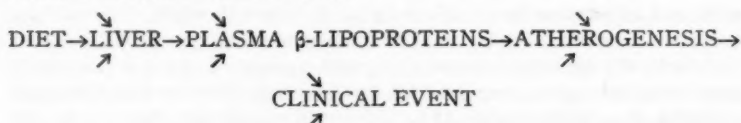
The data to be reviewed pertinent to this hypothesis will deal with various segments of the sequence presented. Nutritional, biochemical, and epidemiological studies will be cited.

Effect of diet upon lipide transport, serum lipides and lipoproteins in animals.—It is beyond the scope of this paper to review the outlines of normal fat metabolism although it is clear that the effects of dietary constituents upon serum lipide and lipoprotein levels must be related to the physiological pathways of fat transport and utilization in the whole organism. Several reviews of the known pathways of fat transport have appeared during the past year (70, 72, 73, 74).

Experimental animals differ widely in their serum lipoprotein content (70) and in the extent to which alterations of dietary fat and cholesterol alter their postabsorptive plasma lipide and lipoprotein patterns: the dog, cat, and rat are very resistant to change while the chick and monkey are less, and the rabbit least. If hypothyroidism and dietary bile acids or dietary bile acids alone are added, all species will respond with some degree of hypercholesterolemia. The particular model in which a dietary effect is studied, therefore, must be rigorously defined, and transposition of the findings to another species such as man must be made with much qualification.

As regards the origin of plasma cholesterol, it is becoming increasingly clear that under physiological conditions the liver is the main, if not the only, source. Tennent *et al.* (75) studied incorporation of acetate-1-C^{14} into plasma cholesterol in the isolated perfused heart-lung-liver preparation. In this system it was found that the liver was essential for the appearance of radioactivity in the plasma cholesterol. Under the unphysiological conditions of intravenous phosphatide infusion, cholesterol may be mobilized from tissues other than the liver (76, 77). Morris *et al.* (78) found that both dietary and endogenous cholesterol contribute to the serum cholesterol in the rat. Under the conditions of feeding 2 per cent cholesterol, approximately 20 per cent was derived from endogenous sources and 80 per cent from the diet, whereas under the conditions of feeding only the tracer (0.05 per cent), approximately 75 per cent was derived from endogenous synthesis and only 25 per cent from the diet. In these experiments there was a negligible change in the plasma cholesterol level. Cholesterol feeding in this species, therefore, appears to retard cholesterol biosynthesis but does not suppress it (79). Absorbed cholesterol appears to be considerably diluted by the endogenous pool of the intestinal mucosa (80). "Isocholesterol," a mixture of 30-carbon

sterols from wool fat containing lanosterol, dihydrolanosterol, agnosterol, and dihydroagnosterol was found to be a less potent inhibitor of cholesterol absorption than β-sitosterol (81). A similar mechanism is postulated for the hypocholesterolemic action of cerebroside-rich extract from mammalian brain (82). Alimentary hyperlipemia was found not to influence the turnover of plasma phospholipides (83).

The type of dietary fat fed to rats appears to have relatively little effect upon serum cholesterol concentrations even though it has been shown to influence the turnover of liver cholesterol. Olson, Jablonski & Taylor (84) found that butterfat, lard, and corn oil fed at 40 per cent (by weight) for three weeks had no effect upon serum cholesterol in the rat. Best *et al* (85) compared a larger number of fats widely differing in iodine number fed at the 20 per cent level and found no effects upon serum cholesterol level in rats. In longer term experiments (11 weeks), Avigan & Steinberg (86) found that 20 per cent corn oil fed at the 20 per cent level to rats gave slightly lower serum cholesterol and higher liver cholesterol ester values than coconut oil. Studies of the incorporation of acetate-1-C^{14} into the liver and serum lipides of these animals showed that more activity was incorporated into the liver and serum cholesterol of the corn oil-fed rats than the coconut oil-fed or control groups. Since the serum cholesterol levels were roughly comparable, these data suggest that unsaturated fat stimulates both cholesterol anabolism and catabolism in the rat. Wood & Migicovsky (87, 88) found similar effects in homogenates of liver from rats fed corn oil and coconut oil. Rapeseed oil, rich in erucic acid, acted like corn oil. Coconut oil was found by these workers to depress cholesterogenesis *in vivo*. In short term (three-day) experiments, Linazasoro *et al.* found that dietary lard, corn oil, cottonseed oil, or hydrogenated vegetable oil at the 15 per cent level were equally effective in stimulating acetate-C^{14} incorporation into liver cholesterol, as compared with animals on a fat-free diet (89).

In view of the growing evidence that the bile acids are the principal pathway of cholesterol excretion in the animal, studies of the effect of diet upon bile acid metabolism are particularly pertinent to an understanding of the regulation of cholesterol and lipoprotein concentration in the plasma. Lindstedt & Norman (90) measured the turnover of bile acids in the intact rat on a commercial diet with cholic acid-24-C^{14} and found an absolute turnover rate of 15 to 20 mg./kg./day. In animals treated with antibiotics to sterilize the bowel, the turnover rate was greatly depressed, presumably because of the lack of microbiological transformations of taurocholate with correspondingly better resorption (91). Portman & Murphy (92) measured the turnover time of cholic acid-24-C^{14} in rats on diets which had previously been shown to alter the output of total bile acids in fistula bile (93). They found that Purina chow caused a relatively high turnover rate of fecal bile acids (36 mg./kg./day) as compared with a purified diet containing sucrose (8 mg./kg./day). The substitution of starch for sucrose or the addition of celluflour to the purified diet increased the turnover rate toward that ob-

served with chow diet and increased the number of chromatographically identifiable bile acids in the feces. High residue diets provide hemicellulose and other substrates for bacteria which apparently modify intestinal flora and correspondingly, the pattern of bile acids excreted. Chicks fed glucose plus cholesterol have lower serum cholesterol values than those fed sucrose plus cholesterol (94), an effect abolished by chlortetracycline (aureomycin). When such birds were given cholesterol-4-C^{14}, the sucrose-fed ones retained more and excreted less label; this effect, too, was abolished by chlortetracycline (95). These data suggest that glucose exerts a flora-modifying effect in the chick not unlike that produced by starch in the rat. These effects of diet may be important in modifying the catabolism of cholesterol and hence influencing cholesterol levels in plasma.

Portman & Sinisterra (96) studied the turnover of cholesterol-4-C^{14} in Cebus monkeys fed for five months diets containing 45 per cent of calories as corn oil, oil plus cholesterol (0.1 gm./100 cal.), or lard plus cholesterol. Their serum cholesterols were, respectively, 237, 268, and 601 mg. per cent. The mean biological half lives for plasma cholesterol were 8.8, 8.4, and 6.6 days for these groups, suggesting that both relative and absolute turnover rates of plasma cholesterol were increased in the lard-fed animals.

Hegsted, Gotsis & Stare (97) have studied the cholesterol-cholate-fed rat as a model for the assay of various fats and oils for hyper- and hypo-cholesterolemic effects. Adult male rats were fed a diet containing 10 per cent casein and the test oils (coconut, butter, tung, cottonseed, margarine, linseed, corn, triolein, safflower, and sardine) at levels of 10 and 20 per cent for four weeks. When the relationship of unsaturation of the oil to serum cholesterol was studied, it was found that oils rich in oleic acid and elaeo-stearic (9,11,13-octadecatrienoic acid) caused the greatest elevations of cholesterol, whereas highly saturated and unsaturated fats both gave lower values, a result at variance with the findings in man (98). When the product (per cent essential "arachidonic plus linoleic" x per cent saturated fatty acid of the dietary fat) was plotted against the mean serum cholesterol, however, a smooth curve was obtained on semilogarithmic co-ordinates. The physiological significance of these observations is not entirely clear. It would appear that the polyunsaturated acids other than linoleic and arachidonic promote hypercholesterolemia in the cholesterol-cholate-fed rat, whereas both saturated and essential fatty acids equally and synergistically counteract this. The responses in this model and in the human seem so dissimilar that they throw doubt on the value of the rat's responses in predicting the effects of fats in man. Leveille & Fisher (99) could not confirm this relationship in the cholesterol-fed chick. The sex differences in cholesterolemia in the rat also appear to be just opposite to what has been observed in man, male rats having lower serum cholesterol levels than females (100, 101, 102).

Although most investigators have been preoccupied with the study of the effect of dietary fat and cholesterol upon serum lipides and fat transport, there has been a growing interest in the effect of dietary proteins and lipo-

tropic factors during the past few years. Handler (103) was among the first to note that choline-deficient rats had serum cholesterol values below the normal range. Ridout *et al* (104) then showed that feeding cholesterol to rats over a 10-week period did not elevate the plasma cholesterol esters unless choline was present in the diet. Wilgram, Lewis & Bloomenstein (105) reported that both the low density and high density serum lipoproteins were decreased in rats fed a high fat choline-deficient diet (106). Olson, Jablonski & Taylor (84) independently noted that young adult rats fed choline-deficient diets developed marked hypocholesterolemia, hypophospholipidemia, hypolipemia, and hypo-β-lipoproteinemia when fed soy protein diets low in methionine and choline. These effects were partially prevented by the substitution of casein for soy protein and completely by the addition of 0.3 per cent choline to the diet. The type and amount of dietary fat in these experiments (butterfat, corn oil, lard, ranging from 6 to 42 per cent by weight) did not modify the effect of choline upon either serum lipides or liver fat, a finding corroborated by Best *et al.* (85). The only difference noted among fats by the latter workers (85) was the propensity for promoting deposition of cholesterol esters in the absence of choline. Olive oil produced the greatest accumulation, coconut oil the least, and sunflower seed oil, lard, and butter occupied intermediate positions. Rosenfeld & Lang (107) have also observed that choline is essential in the rat for the maintenance of normal serum phospholipide values. Wilgram, Lewis & Best (108) noted that feeding of 2 per cent cholesterol to rats did not influence the hypolipemia and hypolipoproteinemia in choline deficiency. This effect has been confirmed by Olson (109) in the cholesterol-cholate-fed rat.

Dietary choline appears to be essential for the maintenance of normal serum lipide levels in all species in which it has been rigorously tested (71, 104, 110). In the Cebus monkey, Mann *et al.* (111) found that hypercholesterolemia from cholesterol feeding was not obtained unless choline was added to the diet.

In the presence of choline, dietary protein appears to exert other effects upon serum lipide concentrations than can be accounted for by its contribution to the "labile methyl" supply. Mann *et al.* (111) found that diets adequate in choline but low in methionine caused the most marked hypercholesterolemia in cholesterol-fed monkeys. Addition of methionine to the diet or substitution of casein for soy protein reduced the cholesterol level. Portman & Mann (93) found that this effect of methionine could be duplicated by cystamine or taurine and appeared to be related to the adequacy of taurocholate formation. Fillios & Mann (112) found substantially the same effect in the rat. Similar effects of methionine-variable proteins upon cholesteremia have been noted in cholesterol-fed chicks (99, 113, 114). Nate, Harper & Elvehjem (115) studied the effects of casein and wheat gluten at different levels in the diet upon the serum cholesterol of the cholesterol-cholate-fed rat. In agreement with other work (112), as the casein level was increased from 6 to 40 per cent, the serum cholesterol levels were reduced;

contrarywise, as wheat gluten was increased from 10 to 68 per cent, there was a progressive lowering of serum cholesterol level, suggesting that amino acid imbalance which may also lead to fatty liver in the rat (116) may also be a critical factor in controlling serum cholesterol values in the rat.

Experimental atherosclerosis.—Experimental atherosclerosis has been produced in an increasing number of laboratory animals by devising dietary and endocrine conditions which markedly elevate plasma β-lipoproteins. During the past year, Hartroft & Thomas (117) reported experiments in which coronary thrombosis and myocardial infarction were produced in rats by feeding a diet high in saturated fat (40 per cent), choline, cholesterol, cholate, and thiouracil for 14 weeks. Serum cholesterols ranged up to 3000 mg. per cent. Eleven to 60 per cent of these animals developed myocardial and renal thrombi and necrosis. Altered blood coagulability and fibrinolysis induced by this regimen may have played a key role in the production of infarctions, since intimal atheromatosis was minimal (118).

Hegsted *et al.* (119) measured the effect of various dietary fats upon aortic sudanophilia in rats fed cholesterol-cholate supplemented diets for 12 weeks. The degree of sudanophilia paralleled the serum cholesterol concentrations which varied widely from 299 mg. per cent (sardine oil) to 1130 mg. per cent (tung oil). Deming *et al.* (120) observed aortic intimal lesions in rats fed cholesterol-cholate-thiouracil diets for 20 weeks. Deoxycorticosterone acetate induced hypertension accelerated the atherosclerotic change. Vitale *et al.* (121) found that dietary magnesium exerted a paradoxical effect upon serum cholesterol and aortic sudanophilia in the cholesterol-cholate-fed rat. High magnesium reduced aortic sudanophilia even though it increased serum cholesterol. I^{131} induction of hypothyroidism in dogs without cholesterol feeding was found to result in sustained hypercholesterolemia and elevated aortic, coronary artery, and hepatic cholesterol levels (122). Lambert *et al.* (123) found that rabbits fed 20 per cent hydrogenated coconut oil developed hypercholesterolemia and atherosclerosis without the addition of cholesterol to the diet. The use of safflower oil as the dietary fat prevented the development of the lesions.

Stamler, Pick & Katz (124) observed that hypercholesterolemia and atherogenesis in cholesterol-mash-fed chicks were aggravated when the protein content of the diet was reduced by supplementation with 45 per cent sucrose. Less dramatic effects of variation in dietary protein and methionine upon hypercholesterolemia and atherogenesis were noted when purified diets were used, although in general the same trend was present. Dicumarol and heparin were found to potentiate atherogenesis (125). Nishida, Takenaka & Kummerow (126) have studied the effect of dietary protein, methionine, choline, and heated fat upon atheroma in cholesterol-fed chicks. They found, in agreement with others, that low protein, low methionine rations in the presence, but not the absence, of choline gave the most severe hypercholesterolemia and atherosclerosis in this species. The substitution of heated for fresh oil depressed the serum cholesterol but did not retard atherogenesis.

Tennent *et al.* (127) found that the degree of atheromatosis of the thoracic aorta and brachiocephalic arteries in groups of cholesterol-fed cockerels correlated best with the log of the mean β-lipoprotein cholesterol. Uhley, Friedman & Ayello (128) found no differences in rates of atherogenesis in "aggressive" and "passive" chicks fed high cholesterol, high fat diets. Rutstein *et al.* (129) found that deposition of lipide in human aortic cells cultured in a medium containing cholesterol (free or protein-bound) was potentiated by stearic and inhibited by linolenic acid.

Effect of diet upon lipide transport, serum lipids and lipoproteins in man.—Several excellent reviews of this subject have appeared during the past year (73, 130, 131). It has been conclusively demonstrated that serum lipides and β-lipoprotein concentrations in man can be altered by dietary changes in (*a*) calories (during periods of adjustment in body composition), (*b*) the amount and quality of dietary fat, and (*c*) the amount and quality of dietary protein. The mechanisms by which these nutrients effect changes in serum cholesterol and lipoprotein concentrations are essentially unknown, but many studies are currently in progress to elucidate these mechanisms. Both the reduction of the percentage of calories supplied by saturated fat and the isocaloric exchange of unsaturated fat for saturated fat have been shown to reduce plasma cholesterol and phospholipide concentrations in human infants (132) and adults (133, 134). Recent work has been devoted to the determination of the specific chemical configurations among the lipides responsible for these effects.

It is now clear that the terms "animal" and "vegetable" do not distinguish between fats which raise and lower serum lipides. Both butter and coconut oil raise serum cholesterol, whereas both corn oil and whale oil lower it (130). Furthermore, the terms "saturated" and "unsaturated" are not suitably descriptive of opposites since neither all saturated fatty acids nor all unsaturated fatty acids are identical in their effects upon serum cholesterol in man (130, 135, 136). The dietary fats containing saturated fatty acids in the range C_6 to C_{14} appear to cause more hypercholesterolemia than saturated fatty acids in the range C_{16} to C_{20} (98, 137). On the other hand, monoethenoid fatty acids such as oleic are not as potent in inducing hypocholesterolemia as linoleic, linolenic, arachidonic, and the highly unsaturated fatty acids of fish oils (138, 139, 140). Finally, the terms "nonessential" and "essential" do not distinguish the serum cholesterol altering capacities of the various polyunsaturated fatty acids. Both linoleic acid (138), which is essential (as previously defined), and linolenic (141) acid which is nonessential have potent hypocholesterolemic properties. Furthermore, the fish oils contain a large variety of polyunsaturated fatty acids which are nonessential and share the 3,6,9 triene moiety (counting from the methyl end) with linolenic acid which are potent in lowering serum cholesterol in man (18, 142, 143).

Keys, Anderson & Grande (143, 144), have proposed a formulation to describe the quantitative effects of variation in the kind and amount of

dietary fat upon serum cholesterol in men who are normocholesterolemic (ca. 230 mg. per cent) on an "average American" basal diet containing about 40 per cent of calories from fat as follows: $\Delta Chol = b(\Delta S) + c(\Delta M) + d(\Delta P)$, where $\Delta chol$ equals change in cholesterol elicited by a change in dietary fat, ΔS equals change in saturated fat, ΔM equals change mono-ethenoid fatty acids, ΔP equals change polyethenoid fatty acids, all fatty acid intakes being expressed in terms of percent of total calories. On the basis of studies involving qualitative comparisons among butterfat, corn oil, lard, olive oil, cottonseed oil, sunflower seed oil, and sardine oil (and change in amount of fat from 9.5 to 41.5 per cent of calories) the coefficients for this formulation were calculated to be: $\Delta Chol = 2.73 \ (\Delta S) + 0.01 \ (\Delta M) - 1.31 \ (\Delta P)$. Since the coefficient for ΔM was insignificant, the simplified formulation became: $\Delta Chol = 2.74 \ (\Delta S) - 1.31 \ (\Delta P)$.

In Keys' hands the formula predicted accurately the effect of all fats tested (except corn oil) in groups of 12 to 20 men, although accuracy of predictions in individuals was much less. Corn oil produced somewhat lower serum cholesterol values (10 mg. per cent) than predicted (145, 146). In effect, this formulation states that the removal of a given amount of saturated fat from the average American diet ($S = 19$, $M = 17$, $P = 4$) has nearly twice the hypocholesterolemic effect as increasing the polyunsaturated fat by the same amount. Substitution of an isocaloric amount of polyunsaturated fat for saturated fat would, by this formulation, give an additive effect, i.e., three times the fall in serum cholesterol. Oleic acid content of the dietary fat appears to be neutral, i.e., neither elevating nor depressing, and this conclusion was verified by Keys and co-workers in a subsequent study (139). Ahrens and associates (136) have criticized Keys' formulation on the basis that it does little more than represent a regression of Δ-cholesterol against the iodine number of the dietary fat. Using O (oleic) instead of M, and L (linoleic) instead of P, to represent the per cent of these acids in a given dietary fat, Ahrens showed that the iodine number of a dietary fat correlated directly with the terms $O + 2L$, $O + 2S$, and $S - L$, and he pointed out that Keys' term $2.74S - 1.31P$ represents only a small departure from the theoretical $S - L$. This, he argued, should not be construed to mean that monoethenoid fatty acids are lacking in effect, particularly since the available oils do not feature the requisite spread in S, M, and P content to test the hypothesis. Although Ahrens' view has merit, it does not disprove the position of Keys. Further, the terms in the equation derived by Ahrens denote per cent of fatty acid in the fat fed at a constant percentage of calories (the simplest case), whereas the terms in Keys' equation denote fatty acids present as per cent of total calories in the diet, so that the latter provides more degrees of freedom for experimental verification than the former. Horlick & Craig (137) found that serum cholesterol was depressed equally by removing animal fat or substituting corn oil or ethyl linoleate to young adults, a finding not entirely in accord with Keys' formulation. Armstrong *et al.* (147) found that adding 57 gm. of corn oil, olive oil, safflower

oil, or butterfat to the "normal" diet of young adults significantly altered serum cholesterol values. Safflower and corn oil were equivalent in lowering serum cholesterol ca. 20 mg. per cent, despite moderate weight gain (one to two pounds in nine days). Olive oil, rich in triolein, had about half the depressant effect of corn oil, and butterfat had no effect.

Tobian & Tuna (148) noted a 10 per cent drop in serum cholesterol in 23 coronary patients given 35 gm. of corn oil daily and asked to "cut down" on saturated fats for one year. Labecki and his associates (149, 150) have reported that combined daily therapy of outpatients with coronary artery disease with a lipotropic supplement and 3 gm. of safflower oil resulted in a significant change in α-β-lipoprotein ratios. Only six out of 25 patients showed a decrease in serum cholesterol over a 30-week period, and no untreated controls were followed for the same period. The supplements given separately were inactive.

Beveridge and co-workers (151, 152) have continued their study of various molecularly distilled fractions of corn and butter oil upon serum cholesterol in young adults. They have found that the most volatile fraction of each oil containing the respective nonsaponifiable fractions has the most potency in raising (butter) or lowering (corn oil) serum cholesterol. An effect of corn oil on serum cholesterol in man out of proportion to the effect of its constituent fatty acids has also been claimed by Grande, Anderson & Keys (145), but not by Ahrens $et\ al.$ (153), who believe that the effect of corn oil can be fully explained by its constitutent triglycerides.

The effects of dietary protein upon plasma lipides and lipoproteins in man are considerably less well understood than the effects of dietary fat. Keys & Anderson (154) could demonstrate no effect of dietary protein in the range of 64 to 138 gms./day upon the serum cholesterol levels of adult schizophrenic men. Kempner (155) and others (156, 157) have observed marked hypocholesterolemia in patients fed the rice diet, which is low in protein (25 gms.), choline (0.2 gm.), and fat (5 gm.). Since this effect upon serum cholesterol is out of proportion to effects observed on subjects fed diets low in fat but replete with protein (144), Olson and co-workers (71, 158) were stimulated to undertake a series of studies of the effect of low protein, high fat diets upon the serum cholesterol and lipoproteins in man. Middle-aged human subjects were fed a control ration containing 100 gms. of protein, 80 gms. of fat (Iodine Number 40) and 350 gms. of carbohydrate for periods of 1 to 2 weeks. Isocaloric substitution of carbohydrate for 75 gms. of protein, yielding a diet containing 25 gms. of vegetable protein, resulted in an average fall in serum cholesterol of 44 ± 4 mg. per cent over a 1 to 2 week period; the fall was sustained as long as ten weeks. Serum β-lipoproteins of the Sf_{0-12} class decreased about 25 per cent with lesser changes in the lower density groups Sf_{12-400}. Upon re-institution of the control diet, the cholesterol and lipoproteins promptly returned to normal or hypernormal values. Furman, Howard & Norcia (159) have reported that isocaloric substitution of carbohydrate for all of the protein in formula diets fed to adult

males caused a marked decrease in serum cholesterol and β-lipoproteins, which was accentuated by the simultaneous administration of the androgen methyl testosterone. In weanling children, protein malnutrition (kwashiorkor) results in marked hypocholesterolemia (160, 161), which is corrected by feeding fat-free milk powder (160, 162).

Our understanding of the mechanisms by which various nutrients alter serum lipide and lipoprotein concentrations in man is rudimentary. It would appear from a study of the know pathways of fat transport that the organ primarily involved in these effects is the liver (70). When dietary fat is lowered without change in saturation, β-lipoproteins in the range Sf_{0-12} decrease in concentration, possibly because of attenuation of cholesterol synthesis (54) or storage (163), but low-density triglyceride-rich β-lipoproteins Sf_{20-400} markedly increase (164). When saturated fatty acids are replaced by polyunsaturated fatty acids in the diet, the fatty acids of cholesterol esters, phospholipides, and triglycerides become more unsaturated (58, 165, 166), as the total cholesterol level falls. This is accompanied by an increased excretion of bile acids in fistula bile (167) and bile acids (168) and sterols (169) in the stool. As regards the effects of dietary protein upon serum lipides, it would appear from studies in both animals (84, 108) and man (71) that choline is essential for the secretion of β-lipoproteins by the liver. Whether specific amino acid imbalances play an additional role in suppressing biosynthesis of lipides or secretion of them by the liver requires further study (115).

Biochemical pathology.—Page (170) has reviewed the multiple factors involved in atheroma formation. The belief that the lipide which does accumulate in the atheroma is largely derived from the plasma is based upon (a) the isolation of serum β-lipoproteins from atheroma in man (171), (b) the chemical similarity between the lipides in the plasma and those in the plaques (172), and (c) the transfer of isotopic cholesterol from serum to atheromatous plaques in man (173). The question of the identity of plaque lipides with serum lipides has been re-examined in the past year with countercurrent distribution, alkali isomerization, silicic acid column and gas phase chromatography. Tuna, Reckers & Frantz (174) studied the fatty acids of the total lipides and cholesterol esters of plasma obtained from "eight normal persons" and those from atheromatous plaques obtained at autopsy. No qualitative and gross quantitative differences were found between the total fatty acids of normal plasma and atheromatous plaques although there was somewhat more linoleic and less oleic acid in the cholesterol esters of plasma as compared with atheroma. Fatty acids varying from C_{12}-C_{22} were demonstrated with double bonds varying in the C_{16}-C_{22} group from one to six. Luddy, Barford & Riemenschneider (175) compared the lipides of plasma obtained from young men with those obtained from atheromatous plaques from old men. The plaques appeared to have more free, and less ester cholesterol and phospholipide than did

the normal plasma. Furthermore, the fatty acids obtained from the ester cholesterol contained less dienoic, less saturated, and more oleic acid than did the control plasma. Similarly, Lewis (166) found less linoleic and more saturated plus oleic acid in plaques than in plasma of patients with clinical coronary disease.

Further work is needed to test the hypothesis that the lipides of atherosclerotic plaques is derived principally from the serum. It may be that only β-lipoprotein lipide exchanges with plaque lipide, which would result in the deposition of less cholesteryl linoleate in the plaque (176). Furthermore, in the work cited, the plasma used as the basis of comparison was not derived from a group identical with the autopsied patients from whom the plaques were obtained.

Epidemiology.—Epidemiologic studies of diet and heart disease in widely varying socio-economic, racial, and national groups have continued, in general, to support the hypothesis that populations which have high rates for coronary artery disease have high mean serum cholesterol and β-lipoprotein concentrations and eat diets which are rich in animal protein, fat and calories (177). Yerushalmy & Hilleboe (178) and Yudkin (179) have presented data which suggest that there is a stronger association between dietary animal protein intake and mortality from coronary heart disease than between dietary fat intake and that mortality. The association between animal protein intake and mortality for coronary disease may in part reflect the association of relatively saturated fat with most animal protein (180) as well as a possible "protective" effect of animal protein-lack at the low end of the scale (71). A relative lack of association of dietary fat intake with coronary morbidity and mortality appears at high levels of fat intake. Bronte-Stewart (130) has shown, however, that if one plots the hard fat liquid fat ratio (as a crude index of relative saturation) against coronary mortality in males 55 to 59 years of age for countries consuming between 100 and 140 gm. fat per capita per day, a remarkable parallelism emerges.

Study of populations living with contrasting dietary patterns in the same community has shown that dietary pattern correlates closer with serum cholesterol level and incidence of coronary artery disease than any other environmental variable. The study of Bronte-Stewart, Keys & Brock (181) of the Bantu, Cape colored, and Europeans living in Capetown, South Africa, is now well known. Toor *et al.* (182) have made similar observations on the mixed population of Israel. Gupta, Iyer & Nath (183) have found that patients with coronary artery disease in New Delhi have significantly higher serum cholesterol levels than healthy urban Indians matched for age and occupation. Groen (184) has noted that Trappist monks who are strict vegetarians have lower serum cholesterol than Benedictine monks who eat a more varied diet. Keys and associates (185, 186) have shown that Japanese living in Japan, Hawaii, and Los Angeles have serum β-lipoprotein cholesterol values and coronary mortality in proportion to their intake of fat

calories, which were respectively 13, 32, and 40 per cent. Studies in Finland (187, 188) have revealed a high mortality and morbidity rate from coronary artery disease in that country, a high mean serum cholesterol (260 mg. per cent) and a diet with 35 per cent of calories from fats, most of which is saturated.

Coronary artery disease appears to correlate better with serum β-lipoprotein concentrations and dietary fat intake than other forms of atherosclerosis, suggesting that thrombogenesis, which appears to be particularly important in initiating coronary occlusion, may be a more critical variable (189, 190) than other factors. Possibly thrombogenesis and fibrinolysis are adversely affected by the same environmental factors, although this is a highly controversial matter (191, 192, 193). Laurie & Woods (194) have reported that although coronary disease is a rarity in the Bantu, aortic atherosclerosis is not uncommon. Kallner (195) has noted that the epidemiology of cerebral and of coronary atherosclerosis in various population groups in Israel are quite distinctive. Mann (189) recommends a healthy scepticism in using vital statistics as a base for most epidemiological studies in this field and believes that the data on the incidence of coronary artery disease and food intakes are so inaccurate as to make a definite test of the coronary-fat hypothesis very difficult at this time.

FUNCTION OF FAT-SOLUBLE VITAMINS

In contrast to the well-known coenzymatic function of many of the B vitamins, the biochemical functions of the fat-soluble vitamins are essentially unknown; correspondingly, their functions are still described in biological rather than biochemical or enzymatic terms. The evidence is growing, however, that all of the fat-soluble vitamins are integral parts of tissue lipoproteins and may have specific structural and metabolic functions as part of the intracellular particulates.

Vitamin A

Dowling & Wald (196) have studied the interrelationship between vitamin A stores in liver and the rhodopsin and opsin content of the retina in vitamin A-deficient rats. They observed that four weeks were required to deplete the liver of vitamin A, whereupon blood vitamin A and rhodopsin levels began to decline and "night blindness" was detected by electroretinography. Two weeks later the concentration of opsin, itself, began to decline and it was concluded that this decline signaled the onset of general tissue protein depletion in the animal. Vitamin A acid (197) is able to function as vitamin A for the extravisual functions of the vitamin (198). Wolf et al. (199) found that the depression in gluconeogenesis in vitamin A-deficient rats previously noted by them (200) results from a diminished glucocorticoid secretion by the adrenal cortex, histological evidence for which was previously noted by Lowe and co-workers (201). Cowlishaw et al. (202)

studied the intracellular distribution of vitamin A in chicken liver. Of the 2300 μg. per cent of vitamin A present, 21 per cent was found in the nuclei, 7 per cent in the mitochondria, and 72 per cent in the supernatant fluid. The per cent for the supernatant fluid is in agreement with the findings in the rat (203, 204). Krishnamurthy, Mahadevan & Ganguly (205) have studied the extent to which vitamin A alcohol and vitamin A ester are liberated by protein denaturants from rat liver cytoplasm and conclude that these two forms of vitamin A are bound to different proteins, as also appears to be the case in plasma (206).

Vignais (207) found that transhydrogenase activity of liver mitochondria was reduced to one-third of normal in vitamin A-deficient rats although DPNH-cytochrome-c reductase remained the same and TPNH-cytochrome-c reductase was doubled. Redfearn (208), however, observed a decrease in succinate-cytochrome-c reductase in liver homogenates from vitamin A-deficient rats, despite an increase in total oxygen consumption. Ubiquinone first detected in liver by Festenstein et al. (209) was found to be consistently elevated above normal in liver from vitamin A-deficient rats (210, 211), but not in vitamin A-deficient chicks (212). Ubiquinone is thought to be closely related to, if not identical with, Q_{275} (coenzyme Q) (213, 214), which participates reversibly in the oxidation of succinate by mitochondria from beef heart.

Vitamin D

Vitamin D promotes calcium absorption, calcification of bone and citratemia, and corrects the existing hypocalcemia, hypophosphatemia, and hyperphosphatasemia in the rachitic animal. Nevertheless, its biochemical function has proved elusive.

De Luca, Gran & Steenbock have continued their studies (215) of the effect of vitamin D upon the oxidation of citric acid by rat kidney tissue. The addition of vitamin D to a nonrachitogenic as well as rachitogenic diet resulted in a depression of citrate and isocitrate oxidation by kidney, but not by liver mitochondria (216). This may account, in part, for the increases in serum and bone citrate found in rachitic animals treated with vitamin D, although similar changes are noted following starvation of rachitic rats (217, 218). Harrison, Harrison & Park (219) have shown that cortisone prevents the rise in serum citrate ordinarily seen when rachitic rats are treated with vitamin D, although the effects of the vitamin upon serum phosphorus and osteoid tissue are not suppressed. This selectivity in the antagonism of cortisone to vitamin D suggests that it does not act as an antimetabolite (220).

Vitamin E

The diversity of the biological manifestations of vitamin E deficiency and the extent to which substances chemically unrelated to vitamin E substitute for the vitamin in various test systems have complicated the delineation of a single role for α-tocopherol. In general, the manifestations of vitamin E

deficiency reflect loss of the integrity of membranes [hepatic necrosis (221), hemolysis of red blood cells (222, 223), exudative diathesis (224), increased postmortem autolysis (225)] and particulates [depressed oxidative phosphorylation (226, 227), decreased lipogenesis (228, 229), respiratory decline (230), and altered DNA/RNA ratios (231)] and loss of antioxidant capacity [peroxidation of body fat, decreased vitamin A storage, ceroid formation and brownish discoloration of uterus (232)]. More complex phenomenon, such as muscular dystrophy and impaired reproductive capacity, may result from a summation of these individual effects. It is of interest that some of these manifestations of vitamin E deficiency are also noted in EFA deficiency.

Electron transport.—Nason and co-workers believe that α-tocopherol participates in electron transport in mitochondria just prior to cytochrome-c (233, 234). They have shown that isooctane extraction of rat muscle mitochondria preparations inactivate DPNH-cytochrome-c reductase and that this activity can be restored by α-tocopherol or by the lipide extract. Upon purification, the "lipide co-factor" proved to be a mixed triglyceride of stearate, palmitate, and oleate. Its activity was 20 times that of α-tocopherol in restoring DPNH-cytochrome-c reductase activity to extracted enzyme; Nason explained this result on the basis of "liberation of α-tocopherol present in the enzyme preparation to active sites." Upon repeated extraction and aging of preparations from both skeletal and cardiac muscle, a greater removal of α-tocopherol and "α-tocopheryl quinone" was accomplished, and under these conditions only α-tocopherol sufficed to restore enzymatic activity. Bouman & Slater (235) found 1.5 µg./gm. protein of "total" tocopherols in the Keilin-Hartree heart muscle preparation (sarcosomes), of which only 0.4 µg./gm. were free and represented all of the tocopherol present in the heart. Cowlishaw *et al.* (202) on the other hand found only 22 per cent of the α-tocopherol of chicken liver in the mitochondrial fraction. Deul, Slater & Veldstra (236) found that isooctane extraction of heart muscle preparation resulted in inactivation of DPNH-cytochrome-c reductase which could be restored by α-tocopherol or vitamin K_1 (237). Marinetti *et al.* (238, 239, 240) have studied the lipides from several purified cytochrome preparations devoid of α-tocopherol and found that some reactivate succinate-cytochrome-c reductase.

No evidence was found by either Nason & Lehman (241) or Slater and co-workers (236) that α-tocopherol could undergo reversible oxidation-reduction to tocopheryl quinone. Crane *et al.* (213) reported the isolation of a quinone from beef heart mitochondria (Q_{275}) which did undergo reversible oxidation-reduction during electron transport from succinate to cytochrome-c and was extractible from various mitochondrial preparations by organic solvents with loss of enzymatic activity which could be restored by the addition of Q_{275}. This compound appears to be closely related to, if not identical with, the ubiquinone of Morton and co-workers (214, 242). The chemical structure of this quinone has been shown to be a 2,3-dimethoxy-5-

methylbenzoquinone with a C_{50} isoprenoid side chain (10 double bonds) at position-6 (243 to 246). It is closely related to both α-tocopherol quinone and to vitamin K_1 as shown in Figure 2. Whether coenzyme Q_{10} is synthesized in the animal body or derived by partial synthesis from microbial sources which apparently synthesize homologues with shorter isoprenoid side chains (243) is not yet determined .

The identification of coenzyme Q (ubiquinone) as an important functional constituent of mitochondria in liver, heart, and kidney led Slater (247) to re-examine his analytical data for free and "total" tocopherol (α-tocopherol + α-tocopheryl-hydroquinone + α-tocopheryl-quinone) in heart muscle. The latter was determined by HCl-ascorbic acid reduction of lipides present in the nonsaponifiable fraction followed by application of the Emmerie-Engel color reaction. It was shown that the increment found after reduction was caused by the partial reduction of ubiquinone. On this basis it would appear that α-tocopheryl quinone is not a physiological constituent.

Interrelationships with antioxidants.—Draper, Goodyear, Barbee & Johnson (248) found that vitamin E-deficient diets supplemented with N,N-diphenyl-paraphenylenediamine (DPPD) or methylene blue sustained reproductivity in 60 females through two reproduction cycles. Reproductive failure on the basal regimen could be partially restored by the administration of DPPD. Butylated hydroxytoluene was inactive under these conditions. It was concluded that DPPD must activate or protect residual stores of α-tocopherol in the rat. Sharman & Moore (249) found that DPPD prevented brown uterus, degeneration of the testis, and abnormal liability of erythrocytes to hemolysis in animals fed vitamin E-deficient diets. Shull, Ershoff & Alfin-Slater (250) reported that supplementation of the vitamin E-deficient diet of guinea pigs with DPPD and certain other antioxidants delayed the onset of dystrophy symptoms but did not prevent the usual rise in plasma and muscle cholesterol.

Interrelationships with cystine and selenium.—Dietary hepatic necrosis in the rat is a nutritional disease of multiple etiology involving at least three factors: vitamin E, cystine, and Factor 3, a nonlipide organic substance whose active constituent has been identified by Schwarz & Foltz (251) as selenium. Inorganic salts of selenium, such as selenite and selenate, were found to replace Factor 3 in the diet. Schwarz & Foltz (252) found that the ED_{50} (dose to protect 50 per cent) for α-Factor 3 from pork kidney powder was 0.72 μg. per cent. Sodium selenite, selenium dioxide, sodium selenate, selenocystine, selenocystathionine, and selenomethionine were essentially equivalent and had an ED_{50} of from 2 to 3 μg. per cent. Certain organic compounds such as dibenzyldiselenide and 2,4-dinitrobenzene seleninic acid were active at about the same level as selenite, whereas other organic compounds such as seleno uracil and octofluoroselenophane were inactive. Inorganic selenium had a very low level of activity with an ED_{50} of 320 μg. per cent.

Patterson, Milstrey & Stokstad (253), Reid *et al.* (254), and Schwarz
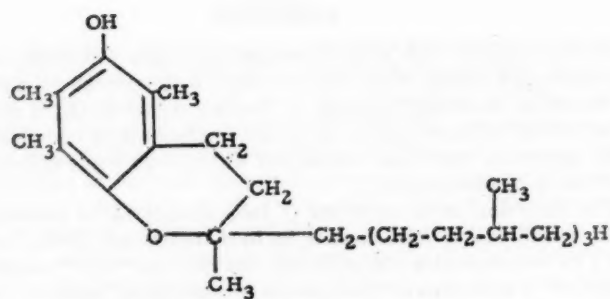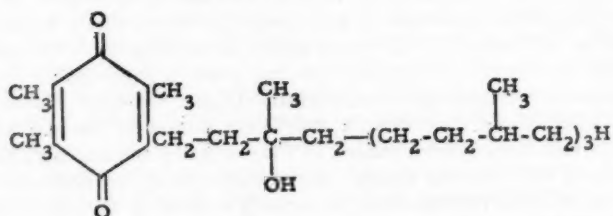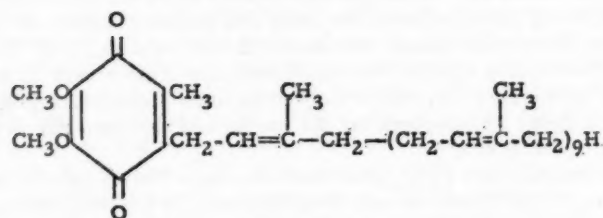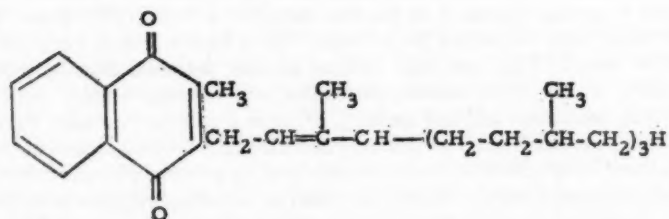
Fig. 2. Structural relationships (vitamin E).

et al. (255) found that selenium was effective in the prevention of exudative diathesis in chicks fed diets containing torula yeast or other vitamin E-deficient substances. High doses of selenium (500 μg. per cent) partially prevented the white striation of breast muscle which responds separately to vitamin E or 0.5 per cent cystine (256, 257). The amount of selenium required to protect against exudative diathesis in the chick is 2 to 5 times that required to protect against hepatic necrosis in the rat. The claim by Schwarz & Foltz (258) that the selenium content of cystine accounts for all of its protective activity against hepatic necrosis in the rat has been denied by Yang, Riegl & Olson (259). Biological functions of vitamin E for which selenium cannot substitute include muscle dystrophy in rabbits (260 to 264), reproductive failure in rats (232, 262), encephalomalacia in chicks, decreased vitamin A storage, incisor depigmentation, peroxidation of body fat, and increased hemolysis of red cells in the rat (232).

Vitamin K

The precise mechanism by which vitamin K controls prothrombin synthesis is unknown. Vitamin K, like vitamin E, has been implicated in electron transport. Martius (227) found a definite increase in P/O ratio after addition of vitamin K_1 to vitamin K-deficient chick liver mitochondria, metabolizing β-hydroxybutyrate, whereas menadione was found to uncouple the same system (265). Vitamin K_1, vitamin K_2, phytol, squalene, and menadione have been shown to be able to reactivate isooctane extracted succinic acid-cytochrome-*c* reductase preparation from heart muscle (237, 266). Bishop has shown (267) that vitamin K_3 (menadione) can restore the photochemical activity of hexane-extracted spinach chloroplasts. D-L-Methionine appears to augment the effect of vitamin K upon coagulation time (268) in chicks.

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MINERAL METABOLISM[1]

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In the five years since mineral metabolism was last reviewed in this journal (1), great advances have been made and a vast literature has accumulated, ranging from the fundamental to the intensely practical, from basic investigations of the metabolic behaviour of minerals to applied studies of deficiencies, toxicities, and practical control procedures. Clearly, all these aspects of mineral metabolism cannot be dealt with, even superficially, within the confines of a single article of acceptable length. Fortunately, the principles and techniques of radioactive isotope usage in mineral studies are so well documented by Comar (2) and others (3 to 7) that they need not be considered further; a book on trace elements in human and animal nutrition has appeared [Underwood (8)]; and a comprehensive monograph on minerals in pastures, in relation to animal health, has been produced [Russell & Duncan (9)]. Critical and informed reviews of iodine metabolism (10, 11) and of iron metabolism (12, 13, 14) have also appeared recently and these minerals will, therefore, not be dealt with specifically here.

CALCIUM AND PHOSPHORUS

Absorption.—The availability of the calcium and phosphorus in feeds and in inorganic supplements to these feeds has attracted increased attention since advances in radioactive procedures have permitted differentiation between the unabsorbed fractions and the absorbed and re-excreted fractions of the feces. These procedures allow "true" digestibility or absorption to be estimated with some confidence and without many of the disadvantages of more conventional methods (15, 16). But the very convenience of radioactive procedures has resulted in a tendency to ignore or gloss over factors which limit their quantitative usefulness. The likelihood of serious overestimation of the fecal output of endogenous calcium and phosphorus has been pointed out by Moore & Tyler (17). It is apparent from their work with pigs that, since the contents of the intestinal tract contained inactive exogenous calcium and phosphorus, the specific activity of the calcium and phosphorus secreted into the upper small intestine would be considerably greater than the specific activity of these elements subsequently reabsorbed from the lower small intestine. This implies that the reabsorption of the secreted

[1] The survey of literature pertaining to this review was concluded in June 1958.

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calcium and phosphorus would be more efficient than would be indicated by the reabsorption of Ca^{45} and P^{32}. These workers point out further, and attention has been drawn to the same point by Tillman & Brethour (18) in relation to phosphorus, that the rapidity and extent of exchange between the Ca^{45} and the P^{32} in the tissues of the gastrointestinal tract and the inactive calcium and phosphorus in the contents of the tract may be a factor of significance. Dietary phosphorus may, in fact, exist in forms not readily exchangeable with P^{32}. If this is so, and some data indicating limited exchange with phytin phosphorus are presented by the latter workers, then true digestibility figures would certainly not be accurate, since they would be based more on absorption data for the radioactive phosphorus than on data for the stable element of the feed. Further work is needed to clarify the contribution of interchange to fecal endogenous values.

Comparisons of the net absorption of calcium and phosphorus from the same materials, with the same animals under the same conditions are rare, but it appears that phosphorus is better absorbed from roughages than is calcium. Thus Lofgreen & Kleiber (19) found the true digestibility of the phosphorus of alfalfa hay to be about 90 per cent in sheep; and Wright (20), also working with sheep, found the daily absorption of this element from a diet of hay and concentrates to range from 32 to 102 per cent. This worker suggested that the whole of the phosphorus is, in fact, available but the actual absorption is controlled by other, unspecified, factors. Hansard et al. (21) found the true digestibility of the calcium of three types of hay to be much lower, of the order of 40 to 50 per cent for young, and 30 to 40 per cent for mature cattle. This difference between young and old cattle is in keeping with the earlier finding of Hansard, Comar & Plumlee (22) that the efficiency of calcium utilization decreases with age in cattle. Even lower values for calcium absorption from a hay and grain diet by young and old cattle are reported by Lengemann, Comar & Wasserman (15). They demonstrated the marked effect of milk in increasing calcium absorption with young and old rats and cattle but found no such effect in rabbits. In seeking an explanation of the enhancement of calcium absorption by milk, Wasserman et al. (23) confirmed the effect of lactose and showed further that L-lysine and L-arginine markedly stimulated the absorption of Ca^{45} in the rat, whereas other amino acids were less effective or ineffective. Subsequently, it was shown that in the normal rat the effects of L-lysine and lactose were additive and in the rachitic rat the effects of L-lysine and vitamin D were also additive [Wasserman et al. (24)]. By contrast, neither lysine, arginine, nor skim milk increased Ca^{45} absorption in the rachitic chick, although treatment with vitamin D promoted nearly complete absorption in this species (24). That increased calcium absorption is the major physiological action of vitamin D is shown further by the isotopic studies of Keane, Collins & Gillis (25) with chicks and of Conrad & Hansard (26) with calves. The latter workers showed that massive doses of vitamin D not only increased absorption but decreased endogenous fecal calcium losses and

increased deposition of radiocalcium in areas of new bone growth. The effect of citric and of tartaric acids in promoting calcium absorption in both normal and rachitic rats has also been demonstrated (27).

A series of studies on the availability of calcium and phosphorus from inorganic materials reveals that within-species differences are mostly small and of doubtful significance. This generalization applies to several species and to a wide range of common mineral supplements (21, 28 to 32). An exception is "soft phosphate with colloidal clay," which is significantly less effective as a source of phosphorus than such materials as bonemeal or mono-, di- and tricalcium phosphates (28, 29, 33). The position in regard to chicks has been thoroughly studied and reviewed by Motzok, Arthur & Branion (33), using 27 phosphate supplements, and the need for caution in the interpretation of availability data based on single experiments is stressed, even with replicated groups.

Phytin as a source of calcium and phosphorus.—The relation of phytic acid to calcium and phosphorus metabolism has been further examined by several workers, and its importance in human nutrition reviewed by Harris (34). Studies with boys and with rats have shown that sodium phytate reduces calcium uptake even more than the equivalent amount of natural phytic acid in wheat and oats (35, 36). Gillis *et al.* (37) have provided additional evidence for the extremely poor utilization of phytate phosphorus by birds. Normal chicks utilized the phosphorus of calcium phytate only 10 per cent as effectively as disodium phosphate, and normal turkey poults less than 2 per cent as effectively. Vitamin D-deficient chicks and poults made negligible use of phytate phosphorus but utilized the inorganic phosphorus as well as normal birds. In pigs, appreciable phytate hydrolysis can occur in the stomach as a result of the presence of dietary phytases of dietary origin and of the favourable pH for their action [Moore & Tyler (17)]. Considerable phytate hydrolysis can occur in the large intestine of this species when calcium phosphate is used as the mineral supplement but not when calcium carbonate is used. It is suggested that the higher intestinal pH induced by the latter is not conducive to bacterial hydrolysis of phytate in the large intestine. Subsequently, in a study of the mode of action of dietary beryllium carbonate on phosphorus absorption, Moore & Tyler (38) showed that the reduction in absorption of this element was the result mainly of a decrease in phytate hydrolysis. This is believed to result from both a probable inhibition of cereal phytase and precipitation of beryllium-phytate compounds in the stomach. In sheep, Tillman & Brethour (18) found that calcium and phosphorus were absorbed equally readily from calcium phytate. By assigning a value of 100 for the true digestibility of the phosphorus of monocalcium phosphate, they calculated that the true digestibility of the phosphorus of calcium phytate is about ninety. Since phytin hydrolysis is accomplished largely by phytases of rumen bacterial origin (39), phytin usage in ruminants is not affected by the presence or absence of plant phytases of dietary origin, as it is in nonruminants (40).

Phosphorus requirements.—A critical re-evaluation of the phosphorus requirements of calves, using 12 different response criteria including bone growth as measured by femur and rib autoradiographs, was made by Wise, Smith & Barnes (41). The minimum phosphorus requirements of calves weighing 200 to 275 lb. at 12 to 18 weeks of age were 0.22 per cent of the air-dried ration. These workers point out that a satisfactory margin of safety would still give a figure of only 0.30 per cent, which is appreciably lower than the recommended standards of 0.40 per cent phosphorus. A valuable feature of this study is the comparison of the precision of the response criteria employed. The bone autoradiographs were found to be the most sensitive and accurate, with the smallest coefficient of variation.

Parturient paresis.—Calcium metabolism in relation to parturient paresis (milk fever) in dairy cows has been reviewed by Boda & Cole (42), who contend that this condition is a failure of the normal homeostatic mechanisms (principally the parathyroid glands) to maintain a normal level of blood calcium in the face of the great loss of calcium from the blood to the milk at the initiation of lactation in high-producing cows. The logical approach to milk-fever prevention, they suggest, is either the preparation of these homeostatic mechanisms for optimal activity in advance of the demands of lactation or their temporary substitution by artificial means. The former approach, based upon the use of low-calcium, high-phosphorus prepartal diets, believed to stimulate endogenous parathyroid hormone production before the initiation of lactation, has been shown by Boda (43) to be safe and effective with high-producing Jersey cows. Subsequently, it was shown that the lower the calcium-phosphorus ratios of the prepartum rations the heavier was the deposition of Ca^{45} and the more diffuse its deposition within the bones of cows. Further, those cows receiving the diets with the higher Ca/P ratios had consistently and significantly lower serum calcium values (44). Management difficulties occur with this technique, since such diets require a marked restriction of intake of roughages which are mostly high in calcium and the cows must be individually fed for at least a month before parturition.

Hibbs & Pounden (45) found that milk fever can be largely prevented by massive (30 million units/day) dosage with vitamin D for several days before calving. In this way, the usual fall in serum calcium and phosphorus is prevented. They claim that the protective effect of the vitamin D is to replace the calcium mobilizing action of parathyroid hormone during the critical period at parturition and probably, also, to increase intestinal absorption of calcium. Subsequently, Conrad, Hansard & Gibbs (46) showed that the main effect of feeding such massive doses of vitamin D is to increase the rate and quantity of calcium and phosphorus absorption from, and to decrease their excretion into, the digestive tract. Only a slight rise in serum calcium levels was found, compared with a large increase in absorption and retention. It is sugegsted that vitamin D may have an effect in preventing milk fever additional to that of increasing calcium absorption, since the

preventive effect is limited to about two days or less after the vitamin dosage is terminated, whereas the increased calcium absorption continues after this period.

A detailed study of serum calcium and magnesium and plasma phosphate levels immediately pre- and postpartum in normal parturient cows indicates that parturient paresis is an exaggeration of normal changes at parturition (47). The complexity of the metabolic disorders of dairy cows at or near parturition and the difficulty of identifying any individual disorder from the clinical syndrome alone, that is, without the blood chemical picture, emerge from the work of Swan & Jamieson (48) in New Zealand and of Hallgren (49) in Sweden.

STRONTIUM

Although strontium has been recognized as a trace element in biological materials for many years, the lack of a sufficiently sensitive and precise analytical technique limited studies with this element. A satisfactory method for both strontium and barium, using the technique of activation analysis and with limits of detection approximately 0.04 μg. strontium and 0.1 μg. barium and an accuracy of ±5 per cent, has now been worked out by Harrison & Raymond (50). It is hoped that the availability of this method will stimulate studies of the normal distribution and metabolic movements of stable strontium. Already Bowen & Dymond (51) have determined the strontium and barium contents of a range of English soils and plants; shown that strontium (but not barium) is preferentially absorbed by plants from most soils; and demonstrated remarkably high concentrations of strontium (up to 2.6 per cent dry weight) in certain native plants growing on strontium-rich soils. The strontium content of plants growing on normal soils ranged from 1 to 169 p.p.m. (mean 36 p.p.m.) which is of the order of that found earlier for pasture plants by Mitchell (52), using spectrographic techniques.

As soon as it became apparent that strontium is one of the most abundant and potentially hazardous radioactive by-products of fission piles, strontium metabolism, especially in relation to its chemical homologue calcium, became a fashionable field for study. The world-wide distribution of Sr^{90} has been reviewed by Libby (53) and Kulp et al. (54); strontium-calcium movement from soil to man has been discussed by Comar, Russell & Wasserman (55); the numerous studies of the metabolism of strontium in animals and man have been compared and reviewed, up to 1956, by Comar & Wasserman (4); the placental transfer of calcium and strontium in the rat and rabbit was studied by Wasserman et al. (56). The metabolism of stable strontium in man has been investigated by Harrison et al. (57), who present strontium and calcium balances for a single individual, on a normal diet without added strontium, which indicate an average daily intake (over 8 days) of 1.99 mg. strontium and a total daily excretion of 1.97 mg., of which 1.58 mg. appeared in the feces and 0.39 mg. in the urine. Further studies of this type, on a

range of diets, are needed. Ray *et al.* (58) showed that there is no difference in uptake of Sr^{90} by living and dead bone and that the mobilization of Sr^{90} from the rat skeleton is greatest on low phosphorus diets (59). In studies with mature dogs receiving injections of Ca^{45} and Sr^{89}, Singer *et al.* (60) demonstrated that both isotopes are excreted into all parts of the gastrointestinal tract—about 30 per cent more strontium than calcium. Analyses of bile collected from animals with fistulae showed that the amount of Sr^{89} secreted by this route was about twice that of Ca^{45}. The same dietary factors which enhance calcium absorption, namely, milk, lactose, and the amino acids lysine and arginine, affect the absorption of radiostrontium to approximately the same degree (15, 23, 24).

From these and other investigations, the following general picture emerges: (*a*) strontium and calcium have a similar and interrelated biological behaviour, move similarly in the food chain from the soil to animals and man, and are affected by many of the same variables such as age of animal, diet, and hormones; (*b*) radiostrontium is usually well absorbed by plants, animals, and man and is deposited and retained in bones and transmitted in relatively large amounts to milk and to the developing foetus; (*c*) differential behaviour of calcium and strontium provides a large factor of protection against Sr^{90} as a result of preferential absorption of calcium from the gastrointestinal tract, preferential excretion of strontium, especially in the urine, and consequent preferential utilization of calcium in bone formation and secretion of calcium in milk; and (*d*) possibilities exist for increasing the discrimination against strontium by dietary means. Further studies of the effect of increasing stable strontium and calcium intakes upon radiostrontium retention in man and other species are required.

MAGNESIUM

Magnesium requirements are markedly affected by high levels of dietary calcium and phosphorus and by thyroxine feeding. Vitale *et al.* (61) observed that the growth inhibition produced in rats by adding thyroxine to the diet could be partially overcome by additional dietary magnesium, the amount being related to the amount of thyroxine used, and that the impaired oxidative phosphorylation of mitochondrial preparations from the hearts of the thyroxine-treated animals could be raised to normal by high levels of dietary magnesium. O'Dell *et al.* (62) found that high intakes of phosphorus accentuated symptoms of magnesium deficiency in rats and guinea pigs as much or even more than high levels of calcium. Hyperemia and nervous disorders were not apparent in the deficient guinea pigs, as in the rats, but there was a derangement of the calcification process, expressed as widespread tissue calcification and failure of bones and teeth to calcify normally.

Recent studies of the metabolic disorder of milking cows known as "grass staggers" or "grass tetany" reveal that there are many factors capable of affecting serum magnesium levels in this species. Swan & Jamieson (63) induced clinical grass staggers in cows, accompanied by the usual hypo-

magnesemia, by underfeeding, with or without thyroprotein dosing, or by thyroprotein alone. They contend that hypomagnesemia results from either a negative energy balance or digestive disturbances or both, although it is not clear why either should particularly affect serum magnesium levels. More fundamental work on this problem is clearly needed. This is further emphasized by the results of recent field studies of grass tetany. Bartlett and co-workers (64) showed that heavy nitrogen fertilization of pastures accentuates the problem, because, it is thought, high ammonia production in the rumen reduces magnesium absorption; the significance of the cationic composition of the herbage is revealed by the extensive investigations of Kemp & Hart (65), who found the highest incidence of grass tetany when the ratio K:(Ca +Mg) exceeded 2.2; Hart (66) also reported an increased incidence of grass tetany on pastures high in potassium because of heavy dressings of potash fertilizers. A specific effect of high dietary potassium levels on magnesium metabolism is supported by the finding of a significant depression in serum magnesium levels in sheep fed rations containing 5 per cent potassium bicarbonate (67). Finally, limited success in reducing the incidence and severity of hypomagnesemia in grazing cattle has been reported by the feeding of magnesium-rich supplements [Allcroft (68)] and by the application of magnesium-rich compounds to pasture [Parr & Allcroft (69)]. The latter point out that a pasture magnesium level of the order of 0.4 to 0.5 per cent MgO on the dry basis (i.e., about double the usual level) is necessary if hypomagnesemia is to be controlled with confidence in this way.

SODIUM AND CHLORINE

Evidence that sodium rather than chlorine is the main limiting factor in various salt-deficient diets fed to sheep and cows has been presented. McClymont and co-workers (70) found that the addition of 0.25 per cent sodium chloride to high-grain fattening diets fed to young sheep resulted in increased food consumption, feed efficiency, and body weight gains. Similar responses were obtained from a supplement of 0.37 per cent sodium bicarbonate, and indications were obtained that the sodium requirement of the sheep was greater than 0.06 per cent of the diet, or 0.88 gm. per day. The body weight increases obtained in this investigation were too large and the amounts of salt too small for an appreciable proportion of the gains to be accounted for by hydration of the extracellular fluids as found by Hix *et al.* (71). Aines & Smith (72) obtained striking increases in milk production, body weight, and roughage consumption from the feeding of sodium chloride at the rate of 60 gm. per day to cows rendered salt-deficient. Similar increases were obtained with sodium bicarbonate supplements to give equivalent intakes of sodium, but magnesium chloride supplements at equivalent levels of chlorine were ineffective.

Th effects of high salt intakes have also been studied in several species. McCance & Morrison (73) showed that, in rats subjected to five-sixths nephrectomy to make the concentrating power of their kidneys resemble

that of man, the effects of starvation and partial dehydration were progressively intensified by substituting 1, 2, and 3 per cent saline for the ration of water. The results are discussed in relation to the problem of "castaways" and the conclusion is reached that "everything should be done to prevent men drinking sea-water in any form." McCance & Widdowson (74) showed that the addition of salt to the food of newborn infants and piglets led to an abnormal increase in weight, an expansion of the extracellular volume and ultimately massive oedema, together with a reduction in the catabolism of tissue protein. There was a rise in serum sodium and chlorine but no increase in potassium excretion. They suggest that this syndrome be called "hypertonic expansion of the extracellular fluids" of "hypertonic oedema," and they stress that it is a condition of the very young. In older animals, the administration of sodium salts in excess of the body's requirements calls forth a series of defensive reactions which, among other things, increases the excretion of potassium in the urine. Such a response, and factors influencing it, have been demonstrated in adult rats (75).

A remarkable tolerance of adult sheep for high intakes of sodium chloride in the food or the water supply has been shown by Meyer & Weir (76) and Peirce (77). The former fed female sheep rations with a sodium chloride content of 0.5, 4.8, 9.1, and 13.1 per cent for 253 days. No deleterious effects on growth, fattening, gestation, or lactation were observed up to the level of 9.1 per cent, but at the level of 13.1 per cent, weight loss of the ewes during lactation was increased and fewer lambs were raised. Peirce (77) administered the sodium chloride in the drinking water of well-fed wethers at levels of 0, 1.0, 1.5, and 2.0 per cent for a period of 15 months. A concentration of 1.0 per cent was found to have no adverse effects, but 1.5 per cent was detrimental to a small proportion and 2.0 per cent was detrimental to all sheep. At this level, the food consumption and body weight declined and some animals became very emaciated and weak. The intake of water increased steadily with increasing salt concentration, so that the sheep offered the 2.0 per cent salt water consumed over the entire period more than twice the quantity of those offered rain water.

ZINC

Parakeratosis in swine.—The demonstration by Tucker & Salmon (78) that zinc supplementation cures and prevents parakeratosis in pigs has been amplified and confirmed in many laboratories (79 to 87). From these investigations, it is apparent that: (a) signs of zinc deficiency, namely, subnormal growth, inappetence, poor feed efficiency, and parakeratotic skin lesions, can arise in growing pigs fed rations containing 30 to 40 p.p.m. zinc, or less; (b) these signs can be completely overcome or prevented by zinc supplements at the rate of 40 to 100 p.p.m. zinc and partially overcome by smaller supplements; (c) in most cases, the severity of the symptoms

increases as the calcium content of the diet increases from below the recommended level to about three times this level, i.e., from 0.5 to 1.9 per cent; (d) the concentrations of zinc in certain tissues, namely, liver, bone, kidney, and whole carcass, are significantly lower in zinc-deficient than in zinc-supplemented animals; but no such differences exist in muscle, skin, small intestines, or erythrocytes.

The aggravating effect of calcium is not supported by the findings of Wohlbier & Kirchgessner (88) with growing fattening pigs or by those of Beardsley (87) with baby pigs. In the former case the possibility of extraneous sources of zinc cannot be excluded, since no signs of zinc deficiency were observed in the controls consuming a diet reported to contain 29 p.p.m. zinc, and since in the latter the zinc content of the basal diet was so low (8.6 p.p.m.) that growth and feed intake were already very severely limited. Hallgren & Swahn (85) found citric and acetic acid supplements to be as effective as added zinc and suggested that the protein content of the diet might also be important, while Lewis, Grummer & Hoekstra (84) showed that the method of feeding can be of significance. Zinc and calcium intakes may, therefore, be regarded as primary but not exclusive factors in the parakeratosis syndrome. However, the mode of action of these elements is still not clear, the ultimate biochemical lesions of zinc deficiency have yet to be revealed, and an explanation of the fact that the relative zinc requirement of the pig is apparently at least 10 times greater than that of the rat has not been advanced. Zinc analyses of tissues have so far provided few clues to the sites at which zinc exerts its profound effects, nor have these concentrations been related in any clear way to the levels or activities of enzymes known to be associated with zinc (81). However, total zinc values give no information on possible differences in physiological availability of tissue zinc or on its intratissue or intracellular distribution, any one of which could be important. Moreover, there is some evidence that the zinc concentrations in tissues cannot be reduced below certain limits. Beyond this, the animal reacts to zinc deficiency by a reduction in the size of tissues, i.e., by stopping growth or by death (87). Finally, it must be stated that the suggestion of Lewis and co-workers (83, 84) that excess dietary calcium reduces zinc absorption through binding by calcium phosphates in the intestine is not supported by the work of Beardsley (87). This worker found that calcium reduces zinc retention in the baby pig primarily by increasing its elimination in the urine.

Zinc deficiency in birds.—The nature of an unidentified mineral, contained in natural product ash, shown to be required by chicks fed certain simplified diets (89 to 92) has been explained in part by O'Dell and co-workers (93, 94) and Supplee *et al.* (95), who found some of the diets inadequate in zinc and potassium. O'Dell, Newberne & Savage (94) estimate the zinc requirement of chicks maintained in lacquered batteries to be 35

p.p.m. This requirement is slightly reduced by lowering the calcium content
of the diet from 1.6 per cent to 1.1 per cent but is not increased by raising
the calcium content to 2.1 per cent. Zeigler, Leach & Norris (96) claim that
the requirement of chicks for "available" zinc is 15 to 20 p.p.m. Their data
indicate a requirement for total zinc between 24 and 39 p.p.m. in a purified
soybean protein diet and only about 19 p.p.m. in a purified casein diet, when
both diets contained 1.23 per cent calcium. The main symptoms of zinc
deficiency are retarded growth, poor feathering, poor calcification, skin
lesions, and parakeratosis of the oesophagus (94, 95, 96). Specific involve-
ment of zinc in the processes of keratinization and calcification is suggested
(94). The latter hypothesis is supported by the work of Supplee et al. (95)
with turkey poults and is implied from the earlier finding of Morrison et al.
(97) that an "unidentified" mineral in the ash of crude feeds affects bone
formation in chicks.

   Zinc and male sex function.—Very high concentrations of zinc have been
demonstrated in the prostate glands of the rat, rabbit, and man and in human
seminal fluid and spermatozoa [Mawson & Fischer (98, 99)]. The accumula-
tion and maintenance of zinc in the dorsolateral lobe of the rat prostate is
largely controlled by the male sex hormone, testosterone, and the rate of its
accumulation on young rats is markedly increased by injections of testos-
terone and gonadotrophin [Miller, Elcoate & Mawson (100)]. Gunn &
Gould (101) also reported that the rate of Zn^{65} uptake by this gland was
reduced in rats by castration but that it could be maintained by injections of
testosterone. Miller et al. (102) examined the effects of zinc on the repro-
ductve system of young male rats. They showed that: (a) a marked retar-
dation of body growth and of growth and development of the testes, epidid-
ymes, prostate, and pituitary glands occurs in zinc deficiency, with, in many
cases, severe atrophy of the testicular germinal epithelium; (b) except for
testis growth and development, similar changes occur in animals receiving a
zinc-supplemented diet restricted in amount to that of the zinc-deficient
group; and (c) all the changes produced by zinc deficiency, except the
testicular atrophy, are reversed by supplemental zinc. They subsequently
showed (private communication) that the administration of testosterone and
gonadotrophin will cause proliferation of the tissues of the secondary sex
organs of zinc-deficient rats but will not prevent degeneration of the semi-
niferous tubules. They contend that this degeneration is a specific effect of
zinc deficiency, whereas the failure of development of the secondary sex
organs results from a gonadotrophin deficiency caused by the inanition
induced by the lack of zinc.

   Zinc and metalloenzymes.—This topic has been reviewed by Vallee (103,
104). Carbonic anhydrase is only one of several enzymes that contain zinc
as a structural and functional component. These include: pancreatic
carboxy-peptidase [Vallee & Neurath (105)]; alcohol dehydrogenase of
yeast [Vallee & Hoch (106)]; glutamic dehydrogenase (107); and lactic

dehydrogenase from rabbit muscle and beef heart [Vallee & Wacker (108)].
Terayama & Vestling (109), on the other hand, found that purified rat liver
lactic dehydrogenase did not contain a heavy metal cation when subjected to
spectrographic, x-ray fluorescence, or chemical analysis. It has also been
suggested that the zinc-containing proteins from human leucocytes are
metalloenzymes (110). These interesting findings await confirmation in
other laboratories and have not yet been related to any functional disturb-
ances in zinc deficiency. Earlier findings that zinc can serve as an activator
for alkaline phosphatase have been confirmed (111), and Fleischer (112)
observed that a leucocytic peptidase appears to have zinc as a cofactor.

Zinc metabolism.—Feaster and co-workers (113, 114) observed that
absorption from the gastrointestinal tract of pregnant rats is poor but that
absorbed radiozinc passes readily across the placenta to the fetuses and
through the mammary barrier to the milk. With steers, it was also found
that absorption of zinc is low, only 3 to 10 per cent of dietary zinc being
absorbed. When zinc was administered intravenously, about 20 per cent was
recovered in the feces and 0.25 per cent in the urine; when administered
orally, 70 per cent appeared in the feces and 0.3 per cent in the urine. In a
study of the behaviour of zinc and radiozinc in the rat, Gilbert & Taylor
(115) demonstrated that zinc is almost wholly excreted in the feces, that
zinc in the body is in a state of constant movement, and that zinc, except
that in the bone and hair, exchanges fairly rapidly with the protein-bound
zinc of the plasma. The existence of a large, freely exchanging pool of soft
tissue zinc is postulated.

Zinc toxicity.—The relatively low order of toxicity of zinc has been
confirmed. Lewis, Hoekstra & Grummer (83) observed no toxic effects on
pigs fed 1000 p.p.m. zinc in the form of zinc sulphate over a period of 20
weeks. Mehring *et al.* (116) found no effect on the growth or feed efficiency
of broilers from zinc additions to practical rations as high as 778 p.p.m.
Pensack & Klussendorf (117) showed that supplemental zinc can be fed to
broilers and laying hens without detriment to growth or feed efficiency at
levels as high as 1200 to 1400 p.p.m. At a level of 3000 p.p.m. zinc, either as
the chloride or proteinate, growth is retarded and appetite depressed. Grant-
Frost & Underwood (118) found that, at a level of 0.5 per cent (5000
p.p.m.) supplemental zinc, as ZnO, the growth, feed consumption, haemo-
globin levels, body fat values, and tissue copper concentrations of young
rats were markedly reduced. The addition of copper to the high zinc diets,
at the rate of 0.4 mg. copper as the sulphate per rat per day, restored body
copper values to normal and significantly raised the haemoglobin levels but
did not improve growth or appetite. It was concluded that high zinc intakes
inhibit growth almost entirely by depressing food consumption and that they
inhibit haemoglobin formation by inducing a copper deficiency in the animal,
caused both by reduced copper absorption from the intestinal tract and by
zinc-copper antagonism at the cellular level.

Copper

Biochemistry of copper deficiency.—Substantial advances in our understanding of the mechanisms underlying the remarkably diverse manifestations of copper deficiency [see Underwood (8)] have emerged from recent studies by Gallagher, Judah & Rees (119) with the rat, and by Gubler, Cartwright & Wintrobe (120) with the pig. The most striking enzymological disturbance in the copper-deficient rat and pig is an early and marked loss of activity of the terminal enzyme of respiration, cytochrome oxidase. The former workers, in an extremely comprehensive investigation of enzyme systems within the tissues, also found a loss of activity of the succinoxidase system but this was believed to be secondary to the cytochrome oxidase disturbance, particularly since the activity of succinic dehydrogenase was found to be normal in copper deficiency. Gubler *et al.* observed some loss of liver catalase activity and a decrease in the content of glutathione in the liver, but the activity of butyryl coenzyme A dehydrogenase, shown by Mahler (121) to be a cuproflavoprotein, was not altered in the copper-deficient pig (120). This finding with respect to catalase is not supported by the results of Lahey *et al.* (122), Adams (123), or Gallagher *et al.* (119), all of whom report that copper deficiency does not affect catalase activity. Gallagher and co-workers (119) claim that the loss of cytochrome oxidase activity results from a failure in the synthesis of its prosthetic group, heme a, rather than of its protein component. In support of this argument are their findings that the livers of copper-deficient rats are almost devoid of heme, a, and that protein synthesis is, in general, normal in copper deficiency. It is pointed out that the loss of activity of the cytochrome oxidase system cannot be attributed to a general suppression of the metabolism of iron because there is no loss of the iron-containing enzymes in the tissues in copper deficiency and no significant fall in haemoglobin values until well after the level of liver cytochrome oxidase has dropped by 70 to 80 per cent. Gubler *et al.* (120) also showed that a marked drop in cytochrome oxidase takes place in copper deficiency in the presence of normal or near normal levels of total tissue iron, myoglobin, and cytochrome. It seems, therefore, that copper is specifically concerned in some way in the synthesis of heme a, the prosthetic group of cytochrome oxidase, and that this must be regarded as one of the basic functions of copper.

The anaemia of copper deficiency has generally been considered an iron-deficiency anaemia resulting from a reduced ability of the blood-forming organs to utilize absorbed iron and to mobilize tissue iron. This concept of the role of copper in iron metabolism has now to be modified. Gubler and co-workers (120, 124) have produced evidence that the ability of the copper-deficient pig to absorb iron from the gastrointestinal tract is impaired, and Bush *et al.* (125), from their studies of plasma iron and tissue iron turnover rates, find no evidence that the mobilization of tissue iron is impaired in copper deficiency. They do, however, demonstrate a reduced ability of the

copper-deficient pig to produce haemoglobin and make the highly interesting discovery that the survival time of the erythrocytes of this species is shorter in the copper-deficient animal than normal. It is suggested that copper is an essential component of adult red corpuscles and that a certain minimum of copper must be available both for the production of red corpuscles and for the maintenance of their integrity in the circulation. Copper-deficiency anaemia, according to these workers, results from both a shortened erythrocyte survival time and a limited capacity of the bone marrow to produce red cells. Whether the latter is caused by a deficiency in synthesis of haemoglobin limiting the production of erythrocytes or a deficiency in the production of erythrocytes limiting the synthesis of haemoglobin is not known. However, in the copper-deficient dog the anaemia is characterized by a reduction in the number of erythrocytes and relatively normal red cell indices. The bone marrow is characterized by a defective maturation of the erythrocytic elements and no deficiency in haemoglobin (126). It seems likely, therefore, that copper plays a role both in haemoglobin synthesis and in red cell maturation. The stage in these processes at which copper exerts its effect is not known, although it can hardly be required for protoporphyrin synthesis since the concentration of free protoporphyrin in the red cells of the copper-deficient sheep is higher than normal [Allen (127)].

Copper deficiency in the rat is further characterized by a considerable depression of phospholipide synthesis [Gallagher et al. (119)]. This failure to form phosphatides at a normal rate has been traced to a specific step in the synthesis, that concerned with the attachment of CoA-activated fatty acids to α-glycerophosphate to form phosphatidic acids. Gallagher (128) has discussed informatively the likely relationship of this abnormality and the other major expression of copper deficiency, lack of cytochrome oxidase, to the demyelination characteristic of copper deficiency in the newborn lamb, rarely or never found in other species. He notes the following significant facts: (a) myelin is composed largely of phospholipide; (b) inhibition of cytochrome oxidase activity can lead to demyelination so that a deficiency of sufficient severity will give the same result; and (c) the lamb is singularly susceptible to interference with the process of myelin formation which proceeds most rapidly during the latter part of gestation because, unlike most other species, lambs can be severely deficient in copper in utero and at birth. Demyelination of the central nervous system could occur in the copper-deficient lamb, therefore, as a consequence, first, of a depletion of cytochrome oxidase activity leading to inhibition of aerobic metabolism and, secondly, of a decrease in phospholipide and hence in myelin synthesis, both present at the critical period when myelin is being laid down most rapidly in this species.

Skeletal changes in copper deficiency.—Bone abnormalities have been noted in copper-deficient sheep, cattle, pigs, and dogs [see Underwood (8)], and more recently in chickens [Gallagher (128)]. Microscopic studies with

swine by Follis *et al.* (129) have fully confirmed the earlier findings of
Baxter, Van Wyk & Follis (130) with dogs. A marked failure of deposition
of bone in the cartilage matrix, concurrent with normal growth of cartilage,
occurs in severely copper-deficient animals of both these species. The
changes are similar to those observed in scurvy and contrast greatly with
most deficiency states in growing animals, in which osteoblastic and chondro-
blastic activities normally fail together. It seems that copper and ascorbic
acid have a unique property in common, namely, the ability to interfere spe-
cifically with the functional activity of osteoblasts while not affecting the
integrity of cartilage cells. The precise biochemical lesion involved in this
expression of copper deficiency is unknown. It would be of great interest to
know, as Gallagher has pointed out, the level of cytochrome oxidase in the
ossification centers.

Cardiac myopathy in copper deficiency.—Atrophy of the myocardium
with replacement fibrosis was reported some years ago to be the essential
lesion of a disease of cattle, characterized by sudden cardiac failure, which
occurred on severely copper-deficient pastures in southwestern Australia
[Bennetts *et al.* (131)]. Similar sudden cardiac failure has now been re-
ported in copper-deficient pigs [Gubler *et al.* (120)]. Cardiac hypertrophy is
a prominent feature in these animals—a hypertrophy greater than can be
accounted for by the occurrence of anaemia. It is suggested that the hyper-
trophy takes place in an effort to compensate for the reduction in tissue
respiration which occurs as a consequence of the very marked depletion of
cytochrome oxidase activity in the myocardium of the severely copper-
deficient animal. Presumably, the sudden cardiac failure could result from
any physical stress placed upon such a depleted myocardium.

Keratin synthesis in copper deficiency.—It has long been known that one
of the earliest and most sensitive indications of copper deficiency in Merino
sheep is the production of wool which lacks the normal, well-defined crimp
and is inferior in physical properties. Lee (132) has demonstrated fleece
abnormalities in four British breeds identical with those encountered in
copper-deficient Merinos. The absence of this specific lesion of copper de-
ficiency in British breeds of sheep in other countries remains unexplained.
Marston (133) and Burley (134) showed that copper-deficient wool has
more sulphydryl groups and fewer disulphide groups than normal, indicat-
ing that copper is required for the formation or incorporation of disulphide
groups in keratin synthesis. More recently, evidence has been obtained that
copper deficiency may interfere with other aspects of keratin synthesis,
notably the arrangement of the polypeptide chains. Thus, Burley & de
Koch (135) showed that wool from copper-deficient sheep contains more
N-terminal glycine and alanine and sometimes more N-terminal serine and
glutamic acid than normal wool.

Availability of herbage copper.—The assimilation, retention, and utiliza-
tion of dietary copper are influenced by many factors, among which the

molybdenum status of the diet can assume particular importance. These aspects of copper metabolism are dealt with in the section on molybdenum. An indication that the chemical forms and combinations of copper in foods can also be of significance emerges from the highly interesting findings of Mills (136 to 139) with respect to copper in pasture herbage. Much of the copper in herbage exists in bound form as organic complexes, part of which is readily extractable by water (136). Seasonal variation in the solubility of pasture copper also exists, and evidence has been obtained that the copper of an aqueous extract is more readily utilized than cupric ions by the copper-deficient rat (137). In such aqueous extracts, a small quantity of copper is present as the free ion or as positively charged complexes, but the greater part is in the form of neutral or negatively charged complexes which appear to be stable above pH 2.5 (138). These stable, soluble complexes of copper, freed from ionic copper by treatment with a cation-exchange resin, promote a more rapid response and a greater storage of copper in the liver of the copper-deficient rat than the feeding of equivalent amounts of copper as copper sulphate (139). It is suggested that copper may be transported through the intestinal mucosa not only as ionic copper but in the form of complexes such as those encountered in herbage. Evidence is accumulating from other sources that certain soluble complexes of copper may be more readily transported through biological membranes than the free ion. Thus, Uzman (140) has demonstrated that the urinary excretion of copper in hepatolenticular degeneration is associated with the excretion of specific copper oligopeptide complexes, and Seelemann & Baudissin (141) obtained a greater stimulation of hematopoiesis and growth in the rabbit from the sodium salt of a copper allylthiourea-benzoic acid complex than from inorganic copper.

Comparative biochemistry of copper.—Beck (142) determined the concentrations of copper in the blood and liver of nonpregnant adults of a very wide range of vertebrate species. In most species, the whole blood values were found to lie between 0.5 and 1 p.p.m. Cu, with the highest levels in the pig (1.4 p.p.m.) and the lowest in the domestic fowl and turkey (0.23 p.p.m.). Marsupials also showed low values (0.3 to 0.4 p.p.m.). Trends in blood copper levels were discerned which did not follow the phylogenetic relationships implied in current systems of classification. The concentration of copper in the liver of most species was found to lie below 50 p.p.m. on a dry weight basis, but higher values were consistently obtained for ruminants, ducks, frogs, and certain fish. A significant sex difference was found in one species only, the Australian salmon (*Arripis trutta*). The author suggests that the blood copper range characteristic of a particular species represents the optimum for the physiological requirements of that species and that the high liver copper level characteristic of some species is a result, not of a higher intake or greater absorption of copper, but of a lesser ability to restrict storage in the liver.

Molybdenum

Molybdenum as an essential element.—The original studies identifying xanthine oxidase as a molybdoflavoprotein and demonstrating a relationship between dietary molybdenum intakes and xanthine oxidase levels in the tissues of rats implied that molybdenum is an essential element for this species [de Renzo *et al.* (143); Richert & Westerfeld (144)]. They did not establish this fact, because neither the growth nor the purine metabolism of the depleted rats was affected and there was no improvement in the animals from the molybdenum supplements. Higgins, Richert & Westerfeld (145) subsequently showed that the rat has an extremely low requirement for molybdenum and tissue xanthine oxidase. Rats grew normally, reproduced, accumulated xanthine oxidase in tissues other than the intestine, and oxidized xanthine normally on diets containing only 0.02 p.p.m. molybdenum. Even when tungstate was added to such diets at levels equivalent to a W/Mo ratio of 100:1 or 1000:1, growth and xanthine oxidation were normal. The same low molybdenum diets had no effect on chicks; but in this species the addition of tungstate to give W/Mo ratios of 1000:1 to 2000:1 was found to reduce growth, tissue molybdenum, xanthine oxidase concentrations, and capacity to oxidize xanthine to uric acid. There was also a 25 per cent mortality. Moreover, all of these effects of tungsten were overcome by a molybdate supplement. Of great interest is the further finding that the tungsten affected molybdenum metabolism not by reducing absorption but by increasing urinary excretion of tissue molybdenum. No such effect from tungstate was observed by Dick (146) in sheep, as is mentioned later.

The first report of a growth-stimulating effect from molybdenum in chicks and turkey poults, without the use of tungsten as an antagonist, is that of Reid *et al.* (147). Their purified soybean protein diet was reported to contain 1.0 p.p.m. molybdenum, most of which was supplied by the protein and was presumably largely unavailable, since significant growth increases were obtained from the addition of only 0.0126 p.p.m. molybdenum as molybdate. Leach & Norris (148) were unable to confirm this finding, but they did obtain a growth response from molybdenum with highly depleted chicks from hens fed a special low mineral diet and with chicks fed a purified casein diet containing 0.5 to 0.8 p.p.m. molybdenum when tungsten was added. These studies reveal no clear relationship between total dietary molybdenum and molybdenum response and point strongly to significant differences in the availability of this element from different sources.

A nutritional role for molybdenum in the growing lamb has been demonstrated by Ellis *et al.* (149). Lambs receiving a ration containing 0.36 p.p.m. molybdenum made significantly smaller live-weight gains than control animals consuming the same diet to which molybdate was added to raise its molybdenum content to 2.36 p.p.m. Food consumption on the two diets is not given, but a significant increase in cellulose digestibility from

the addition of molybdenum is reported. The conclusion is reached that molybdenum stimulates the growth of lambs under these conditions by increasing cellulose degradation by rumen organisms. It is suggested, further, that part of the reported beneficial effect of alfalfa ash for ruminants fed poor quality high roughage rations (150, 151, 152) resides in its molybdenum content.

Absorption, excretion, and retention.—The outstanding finding with respect to molybdenum metabolism in recent years is the demonstration by Dick (146, 153, 154) that absorption, excretion, and the route of excretion of this element are markedly influenced in the sheep by the level of dietary inorganic sulphate. This important discovery has been confirmed and extended for the sheep by Scaife (155) and apparently applies also in the rat, judging by the molybdenum toxicity studies of Miller, Price & Engel (156) and Van Reen & Williams (157). This factor can be so potent that nutritional investigations with molybdenum are largely valueless unless the sulphate content of the diet is taken into account. Thus, in one experiment in which sheep were fed a diet containing less than 0.1 per cent sulphate plus 10 mg. Mo/day, 63 per cent of the molybdenum was recovered in the total excreta in a period of four weeks, of which 3 to 5 per cent appeared in the urine, whereas such sheep on a diet containing 0.3 per cent sulphate plus the same amount of molybdenum excreted 96 per cent in the total excreta, of which 50 to 54 per cent appeared in the urine. The actual outputs in the feces were very similar on the two diets [Dick (153)]. Scaife (155) fed sheep a low sulphate and a high sulphate diet plus 50 mg. Mo/day in each case. In the former, only 5 per cent of the molybdenum appeared in the urine, compared with 30 to 40 per cent on the high sulphate diet. Dick (146) showed, further, that the total body molybdenum of sheep maintained for 35 days on a diet supplying 0.3 mg. Mo/day was 93 mg. when the sulphate intake was 0.9 gm./day and 17 mg. when the sulphate intake was 6.3 gm./day. An even more striking effect of inorganic sulphate on molybdenum retention was observed on diets supplying 20.8 mg. Mo/day. The total body molybdenum figures in this case were 298 mg. on the low sulphate and 28 mg. on the high sulphate diet.

Much remains to be learned of the quantitative dietary relationships between molybdenum and inorganic sulphate and of the mechanism of action of the latter, but the following conclusions may be drawn from existing facts: (a) sulphate limits molybdenum retention both by reducing intestinal absorption and increasing excretion, the extent of each depending upon the previous history of the animal in regard to molybdenum and sulphate intakes (146, 155); (b) increased urinary excretion of molybdenum is not a passive result of the greater urine volume which occurs on high sulphate intakes (153); (c) the sulphate effect is not shared by other anions tested, notably tungstate, selenate, silicate, permanganate, phosphate, malonate, and citrate (146, 155); (d) sulphate of endogenous origin, e.g., from the oxi-

dation of sulphur-containing amino acids, can be just as effective in promoting urinary excretion of molybdenum as dietary inorganic sulphate. This is implied from the action of orally administered thiosulphate (146, 157), methionine (155, 157, 158), and cystine (157); of the effect of high protein diets (146); and of catabolic breakdown of body tissue (155); and (*e*) the effects of sulphate on molybdenum absorption and excretion can be explained on the hypothesis that inorganic sulphate interferes with and—if its concentration is high enough—prevents the transport of molybdenum across membranes (146).

Molybdenum-copper interrelations.—A reciprocal antagonism between molybdenum and copper first became apparent when Ferguson and co-workers (159) showed that "teart," a scouring disease of cattle caused by excessive intakes of molybdenum, responded to copper sulphate therapy; and Dick & Bull (160) demonstrated a severe limiting effect of molybdenum on copper retention in cattle and sheep. Further evidence of the significance of the molybdenum-copper ratio in the diet to both ruminants and monogastric species has since been obtained [see Dick (154)], but very little light has been shed on the mechanisms of the copper-molybdenum interaction. In fact, the mode of action of copper in dramatically curing the diarrhea and weight loss of high molybdenum intakes is quite obscure. Treatment with copper is not necessarily associated with any marked depression of the molybdenum concentrations in the blood and tissues, nor is the scouring and weight loss necessarily associated with a lowering of the animal's copper status to deficiency levels [Allcroft & Lewis (161)]. Moreover, relatively small amounts of injected copper are remarkably effective in controlling the disease.

The influence of molybdenum on copper metabolism, on the other hand, has been greatly illuminated by the investigations of Dick. In the sheep, molybdenum exerts its effect on copper only in the presence of inorganic sulphate (162). Neither molybdenum nor sulphate alone interferes with copper retention, and the effectiveness of either is increased to a maximum as the intake of the other is increased (163). This means that the copper status of the animal could increase or decrease, depending upon the relation between the copper intake and the intakes of molybdenum and sulphate. Evidence of these different effects has been observed under field and laboratory conditions. Thus, chronic copper poisoning associated with extremely high liver copper levels in the sheep has been observed under conditions of moderate copper intakes and very low dietary levels of molybdenum and sulphate. Conversely, depletion of the animal's copper reserves, even to the extent of clinical copper deficiency, has been reported after several months on normal copper and high molybdenum and sulphate intakes (164). Under conditions of very high intakes of molybdenum and sulphate, the characteristic lesions of copper deficiency in the wool appeared immediately, in spite of the fact that liver copper was not depleted

and blood copper levels were elevated above normal (165). These rather complex findings can be explained on the hypothesis that a membrane whose permeability to molybdenum is impeded or blocked by sulphate, impedes or blocks copper transport, but no evidence of the actual mechanism of the postulated interference with membrane transport of copper has yet been obtained, nor have the possibilities been excluded that in the "physiological" copper deficiency of very high molybdenum and sulphate intakes, the molybdenum fixes the copper in an unavailable form or antagonizes the copper-containing enzymes [Marston (166); Dick (165)]. In regard to the latter, mention should be made of the findings of Scaife (167). He prepared, from sheep's hide, copper-containing proteins that had several properties of true enzymes. They were inhibited by molybdate *in vitro,* and he suggested that molybdenum may also directly inhibit enzymatic activity *in vivo.*

There is evidence also that other dietary factors can exert a modifying influence on the interrelationship between molybdenum and copper. Dick (154) has produced data indicating that high manganese intakes can block or antagonize the limiting effect of molybdenum on copper retention in sheep, even in the presence of adequate sulphate, although no such effect was demonstrated by Mylrea (168) with steers. Dick (154) showed, further, that the addition of manganese and molybdenum together exerts a severely limiting effect on copper retention when sheep are on a high protein diet. These intriguing findings warrant further study, especially as the occurrence of hypocuprosis in sheep and cattle in parts of England and New Zealand cannot be explained in terms of the copper, molybdenum, and sulphate contents of the pastures that the animals graze [Allcroft & Lewis (161); Cunningham (169)]. It is apparent that dietary factors, other than molybdenum and sulphate, can affect copper absorption and utilization either directly or, like sulphate, through an interaction with molybdenum.

Molybdenum toxicity.—Many of the earlier findings on molybdenum toxicity [see Underwood (8)] are of doubtful validity in the light of recent knowledge of the profound effects of copper, inorganic sulphate, and other dietary factors upon molybdenum retention, considered above. Attempts to define the enzymatic defects responsible for the signs of molybdenum toxicity in the rat have so far met with limited success. Van Reen (170) and Williams & Van Reen (171) demonstrated a significant increase in liver alkaline phosphatase activity and a significant decrease in kidney and intestinal alkaline phosphatase activities. This appeared, in each case, to be a reflection of altered synthesis of the enzyme rather than a direct influence of the molybdenum on the enzyme assay system. The inclusion of methionine, cystine, sodium thiosulphate, or sodium sulphate in the diet was subsequently shown to ameliorate both the growth depression and the abnormalities in the activity of the phosphatases of molybdenum toxicity. This was thought to be the result of reduced tissue molybdenum

and was not considered a direct effect of the sulphur compounds on the enzyme levels or activities [Van Reen & Williams (157)]. In a further study, Mills *et al.* (172) found the activity of liver sulphide oxidase to be markedly depressed in molybdenum toxicity in the rat but could detect no change in the activity of liver cysteine desulphhydrase, of kidney aryl sulphatase, or in the oxidation of L-cysteine sulphinate by liver homogenates. Whether the depression in liver sulphide oxidase activity represents the fundamental lesion responsible for growth failure in molybdenum toxicity is not known, but it is clear that a dysfunction of sulphur metabolism which could account for the protective effect of sulphate in the rat was not revealed by these studies. The influence of this ion on the permeability of cell membranes to molybdenum remains, so far, the only explanation of this effect.

<div align="center">COBALT</div>

Cobalt deficiency in ruminants and its relation to vitamin B_{12} have been comprehensively reviewed by Russell & Duncan (9), Smith & Loosli (173), and Underwood (8). Extensive and increased areas of cobalt-deficient grazings have been revealed throughout the world and a unique nutritional situation disclosed—a situation in which ruminants appear to utilize cobalt solely as an integral part of vitamin B_{12} and are directly and completely dependent upon the activities of microorganisms within the rumen for their supply of this vitamin. Cobalt deficiency in these species is therefore essentially a vitamin B_{12} deficiency and can be prevented or overcome by injections of the vitamin in appropriate doses (173). Practical control, however, is normally achieved by supplementation with cobalt, either directly by oral dosing or additions to the feed, or indirectly by treatment of the pastures with cobalt salts or ores. A new procedure for supplying the small amounts of cobalt required by the ruminal organisms to enable them to synthesize the host's requirements for vitamin B_{12} has been evolved by Dewey, Lee & Marston (174). This procedure ingeniously takes advantage of the tendency of heavy, foreign bodies to remain within the ruminant forestomachs. Small dense pellets have been produced, consisting of some 90 per cent cobaltic oxide and having a specific gravity of 4.0 to 4.5. These cobalt "bullets" are delivered into the sheep's oesophagus and lodge in the rumen or reticulum where they usually remain to yield a steady supply of cobalt to the rumen liquor. The prolonged capacity of such pellets to provide the cobalt requirements of sheep on cobalt-deficient fodder has been established, but there is evidence that they can become ineffective when administered to young lambs because of the build-up of a surface coating of calcium phosphate. This coating is reported to be rare in adult sheep, and there is no doubt that such pellets provide a simple and effective means of administering cobalt supplements. Whether the new technique has wider nutritional possibilities has yet to be determined.

SELENIUM

Interest in the biological significance of selenium has shifted from its toxic properties to its role as an essential element, under certain highly specified conditions, in the nutrition of the rat and the chick. This highly interesting work had its origin in the observation of Schwarz (175), in Germany, that diets containing yeast as the primary source of protein produced liver necrosis in rats which could be prevented by supplementary cystine or vitamin E. Subsequent studies in U.S.A. by this worker indicated that: (a) *Torula* yeast diets produced a similar liver necrosis; (b) American brewer's yeast diets not only failed to produce liver necrosis but prevented the necrosis produced by *Torula* yeast diets; and (c) the brewer's yeast contained insufficient cystine or vitamin E to account for its protective action. It appeared, therefore, that there was a third factor, designated "factor 3," present in brewer's yeast that was capable of preventing liver necrosis [Schwarz (176, 177)]. Factor 3 has also been found in casein and a wide variety of natural products (178). Chicks fed *Torula* yeast, vitamin E-free diets were found by Scott et al. (179) to grow poorly and to develop exudative diathesis (but not liver necrosis) which could be prevented by vitamin E or by an unknown factor similar to "factor 3" but not by cystine or antioxidants such as p-diphenylphenylenediamine. Schwarz & Foltz (180) identified "factor 3" as an organic compound containing selenium, and selenite was found to replace it in the diet. The unknown component which promotes growth and prevents exudative diathesis in chicks on *Torula* yeast diet was also identified as selenium by two groups working independently [Patterson, Milstrey & Stokstad (181); Schwarz et al. (182)]. Levels of selenium, as sodium selenite, of approximately 0.1 p.p.m., gave full protection against either the necrotic liver degeneration in the rat or the exudative diathesis in chicks.

Many questions as to the mode of action of selenium in nutrition and its relation to vitamin E remain unanswered. There is no evidence that selenium will replace tocopherol in other functions, such as the prevention of encephalomalacia in chicks or that the need for selenium is completely eliminated by vitamin E. If there is a requirement for selenium in the presence of vitamin E, it must be less than 0.03 p.p.m. for chicks since the *Torula* yeast diet, plus vitamin E, on which good growth and health were maintained was shown to contain 0.03 p.p.m. selenium (181). On this evidence, a deficiency of selenium in the presence of adequate vitamin E seems remote, since cereal grains and other common feedstuffs normally contain appreciably higher selenium concentrations. However, more precise analytical methods for this element are needed so that the distribution of selenium in natural materials grown outside seleniferous areas can be determined more accurately.

Toxic aspects of selenium have not been entirely neglected in the period

under review. Linseed oil meal is markedly superior to casein in protecting rats against selenium poisoning [Halverson, Hendrick & Olson (183)]. Some fraction of the meal, other than protein, is responsible for its protective effect, but neither the nature of the active principle nor its mode of action has yet been revealed. Linseed oil meal does not, however, reduce deposition of selenium in the liver [Olson & Halverson (184)]. As Halverson and co-workers (183) have indicated, these findings point to a need for a reconsideration of protein and other dietary effects in selenium poisoning. In the meantime, linseed oil meal appears to be the supplement of choice in seleniferous areas. Further evidence of the effectiveness of arsanilic acid and 3-nitro-4-hydroxyphenylarsonic acid in counteracting the effects of chronic selenium poisoning in pigs (185) and chicks (186) has been obtained; and other organic arsenicals, notably triphenylarsine, arsenomethane, and p-hydroxyphenylarsonic acid, are effective at a level of 15 p.p.m. of arsenic [Leitis, Palmer & Olson (187)]. The importance of type of diet, breed, and possible unknown environmental differences in determining the response of chicks to arsenic treatment in selenium poisoning has been pointed out by Carlson *et al.* (186).

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OXYGENASES AND HYDROXYLASES[1,2]

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Introduction

An increasing number of biochemical reactions are known to arise from oxygenase activity. The term "oxygenase activity" is used to describe a direct reaction with molecular oxygen whereby one or more oxygen atoms are fixed on the substrate. Aromatic compounds acquire in this way one or more hydroxyl groups. In this case the term "hydroxylation" is more descriptive. Hydroxylation of aromatic compounds prepares the molecules for subsequent dehydrogenation or ring opening. In some cases it leads to the appearance of highly active hormones. It was shown more recently that straight C-chain compounds are also subjected to enzymic oxygenation.

Two main questions may arise concerning oxygenation. One deals with the biological significance of specific (enzymatic) and nonspecific (often nonenzymatic) hydroxylation and oxygenation. We feel that this question cannot be solved until more information is presented concerning the second main question, which deals with the mechanism of oxygenation in general. In the present review we will compare the reaction mechanisms of several types of oxygenation and hydroxylation. Although we are aware that this may lead to additional unsolved questions, we want to show at least just how ignorant we are about this important problem.

Hydroxylation of the Aromatic Nucleus

Tyrosinase.—Many excellent reviews (1, 2, 3) deal with the monophenoloxidase activity of polyphenoloxidase preparations obtained from different sources. It is generally accepted that the reaction with monophenols such as tyrosine or cresol is sluggish and is characterized by an induction period, the duration of which is affected by the presence of reducing or oxidizing agents. It is accepted now that H_2O_2 does not arise during oxidation. Neither should the quinone, corresponding to the diphenol added, act

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[1] The survey of the literature pertaining to this review was completed in September 1958.

[2] The following abbreviations are used: DHFA for dihydroxyfumaric acid; DOPA for dihydroxyphenylalanine; DPNH for diphosphopyridine nucleotide (reduced form); EDTA for ethylenediaminetetraacetate; H_2M for dihydroxy maleic acid; TPNH for triphosphopyridine nucleotide (reduced form); XH_4 for tetrahydrofolic acid.

as an oxidant of the monophenol. The scheme of Lerner (2) satisfactorily explains a number of facts. As an important point, we may note that hydroxylation is brought about within a complex containing Cu^{1+}, tyrosine, and oxygen (Fig. 1).
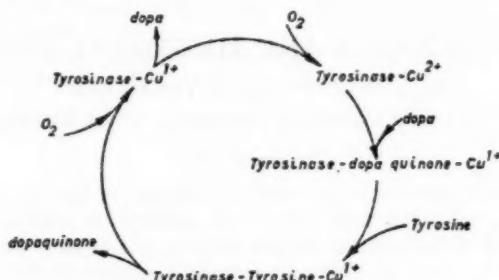
FIG. 1. Hydroxylation of tyrosine.

The intimate mechanism of hydroxylation within the complex might be:

$$\longrightarrow Cu^{1+} + O_2 \; - - - \rightarrow Cu^{2+} + O_2^{1-} \tag{1}$$

$$O_2^{1-} + 2\,Ar \cdot H - 1\,e \; - - - \rightarrow 2\,Ar \cdot OH \tag{2}$$

$$Cu^2 + 1\,e \; - - - \rightarrow Cu^{1+} \; - - - - - \tag{3}$$
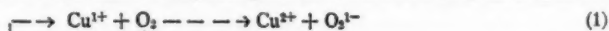
Reaction (1) has been suggested by Baxendale & George for explaining the association of Cu proteins with the respiratory hemoproteins (4). We would stress that this interpretation of the mechanism, the personal view of one of us (Vercauteren), is strictly hypothetical but, as will be shown later, is quite in line with other examples of hydroxylation mechanisms.

The small amount of diphenol added to overcome the induction period is used as a reductans of Cu^{2+}, according to the following reaction:

$$4\,Cu^{2+} + 2\,\text{catechol} \; - - - \rightarrow 4\,Cu^{1+} + 2\,o\text{-quinone} + 4\,H^+ \tag{4}$$
$$4\,Cu^{1+} + 4\,H^+ + O_2 \; - - - \rightarrow 4\,Cu^{2+} + 2\,H_2O \tag{5}$$
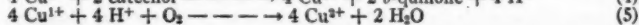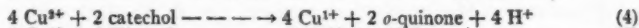
This is the reaction scheme for polyphenoloxidase activity. Reaction (5) may be in competition with reaction (1). Frieden has shown that the reduction by polyphenols of Cu^{2+} is possible (5). Valency changes of copper during the catalysis of melanin formation have been demonstrated by Issaha (6), but Kertesz (7) contests this mechanism as invalid for polyphenoloxidase activity. Instead of this a reaction is proposed between the o-quinone and the monohydroxyphenol.

$$o\text{-quinone} + \text{monophenol} \; \overset{+\,H_2O}{- - - \rightarrow} \; 2\,o\text{-dihydroxyphenol} \tag{6}$$
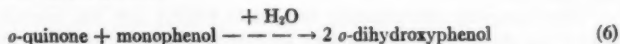
This reaction is catalysed by free and nonprotein-bound metallic ions. A

preparation of Cu-free protein from potato polyphenoloxidase was recombined with various ions. Nonspecific catalysis was evident. Co, Ni, Va, and Cu were effective while Fe, Zn, Cr, Mn, and Mg were inactive. Cu was 2.5 times as active as other cations. Therefore, according to Kertesz, we can hardly accept that fact that we deal with a true monophenolase. On the contrary, a very high degree of specificity was found in restoring polyphenoloxidase activity. Only Cu was effective.

Another point of contested specificity is that of dopa catalysing the oxidation of the corresponding tyrosine. Dopa is said to be superior to all other diphenols tested. But, as appears from the data of Lerner *et al.* (8), the effect of dopa in shortening the incubation period by mammalian tyrosinase is shared to a lesser extent by other structurally related compounds, indicating that a more general explanation than compound specificity should be sought for this phenomenon. The values reported as a per cent of the maximal effect are shown in Table I:

TABLE I

THE EFFECT OF VARIOUS SUBSTANCES ON THE INDUCTION
PERIOD OF TYROSINASE

Substance	Effect on Induction Period
L-DOPA	100
DL-DOPA	90
Catechol	61
Homogentisic acid	47
Hydroquinone	26

A strong argument for the scheme of Lerner is the competitive inhibition of both enzyme activities by N-acetyl-, 3-fluoro- and N-formyl tyrosine (8, 9).

A free NH_2 and OH group is required for enzyme action. This holds for the animal enzyme. For mushroom tyrosinase the situation is different. Only a free OH group is required. *p*-Methoxytyrosine, N-formyltyrosine ethylester and N-glycyl-L-tyrosine react, although slowly. This difference in behaviour is difficult to explain when attachment of the substance to the protein of tyrosinase is not considered.

Enzymatic hydroxylation of phenylalanine to tyrosine.—The work of Kaufman (10, 11) has revealed interesting new insight in the mechanism of hydroxylation. Working with a combination of rat and sheep liver enzyme, he obtained evidence for the role of a labile nonprotein cofactor. This cofactor is probably a pteridine compound, possibly tetrahydrofolic acid (XH_4). 2-Amino-4-hydroxy-6,7 dimethyltetrahydropteridine is also active. Antifollic acid (aminopteridine) is a powerful inhibitor. The scheme Kaufman recently suggested follows:

$$XH_4 + O_2 + \text{phenylalamine} \xrightarrow[\text{enzyme}]{\text{rat}} \text{tyrosine} + H_2O + Y \tag{7}$$

$$TPN^+ \longleftarrow - - - - - - - - - \xrightarrow[\text{enzyme}]{\text{sheep}} - - - - + TPNH$$

TPNH regenerates the oxidized cofactor Y. Anaerobic preincubation shortens the lag period as XH_4 concentration rises. Fe^{2+} is involved in the reaction.

It is still not completely settled whether DPNH can also be used. This system is similar to the one which will be discussed later, where Fe^{2+} is associated with a reducing substance, namely, ascorbic acid. It may be too early to speculate on the role of XH_4 as an intermediate electron carrier, but it seems to us that the main biological interest of the reaction scheme is the association of hydroxylation with TPNH (or perhaps DPNH) oxidation. This means that this system could be one of terminal oxidation in cellular respiration. Further examples will be discussed later. Mitoma et al. isolated a rather similar system from liver microsomes (12). The hydroxylase is less substrate specific than the one discussed earlier. It requires both nicotinamide and TPNH for activity. That α-α^1-dipyridyl and p-chloromercuribenzoate are strong inhibitors suggests that Fe^{2+} and SH groups are involved in enzyme action. It is very striking that not a single natural substrate was hydroxylated by liver microsomes. L-Phenylalanine, L-tryptophan, tryptamine, phenylacetic acid, anthranilic acid, and kynurenine are not attacked. To facilitate comparison with nonenzymatic hydroxylation, we will list the substrates used and their hydroxylation products in Table II.

TABLE II[*]

POSITION OF —OH GROUP ON AROMATIC NUCLEUS OF THE
HYDROXYLATED SUBSTRATE

Substrate	Position in	
	Main Component	By-Product
Acetanilide	4	2 and 3
Aniline	4	
Nitrobenzene	4 (trace)	
Salicilic acid	2 and 5	
Naphthalene	1	
Quinoline	3	6 and 7
	(5 and 8 destroyed)	
Diphenyl	2	4

[*] After Mitoma.

In naphthalene hydroxylation, dihydrodiol naphthalene is an intermediate (13). The mechanism could be as follows in Fig. 2:

Fig. 2. Hydroxylation of naphthalene.

Reactivity is highest with nonpolar substrates, and Mitoma suggests that the primary function of microsomes should be the detoxification of those compounds.

The transformation of p-*hydroxyphenylpyruvate into homogentisic acid.* —For the earlier literature on this subject we refer the reader to the survey of Lerner (2). This author offers an interesting suggestion concerning the mechanism of hydroxylation (Fig. 3):
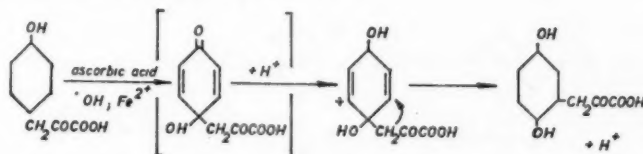
Fig. 3. Hydroxylation of p-hydroxyphenylpyruvic acid.

The data obtained by La Du (14) should not be bypassed without comment. This author shows that catalase is required in this reaction. Ferritin, hemoglobin, or cytochrome-c cannot replace the enzyme. Larger amounts of the enzyme may inhibit hydroxylation. As far as we know, this situation is unique, since in a number of other hydroxylations catalase is described as an inhibitor only. Whether catalase works as a peroxidase or as an hydroxylase is a question which cannot be settled now but deserves attention.

Hydroxylation of dopamine.—The transformation of dopamine (dihydroxyphenylethylamine) into noradrenaline involves a type of hydroxylation we have not dealt with before. The mechanism suggested[3] is that of quinone methine formation (Fig. 4):
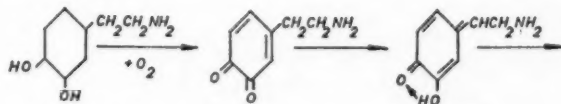
Fig. 4. Hydroxylation of dihydroxyphenylethylamine.

[3] Mentioned in discussion during Colloquium C, Fourth International Congress of Biochemistry.

We see that hydroxyl group arises from oxygen by borrowing a hydrogen, not from the medium as in transformation of *p*-hydroxyphenylpyruvate into homogentisic acid, but from the side chain of the molecule.

Steroid hydroxylation.—11 β-hydroxylation of steroids leads to the appearance of interesting hormonal properties. Hayano & Dorfman (15) have prepared acetone powders from adrenal glands the buffer extract from which, fortified with Mg and fumarate, has powerful 11 β-hydroxylase actvity in the presence of oxygen. One point concerning the possible mechanism of hydroxylation is very conclusive: isotope experiments exclude the direct addition of water in the course of hydroxylation.

According to Tomkins (16), steroid hydroxylation is not fully inhibited by 2.4 moles/ml. of EDTA. This may not be surprising, since metal complexes can be catalytically active (see below). Cofactors are not yet identified. As a hydroxylation mechanism, Tomkins suggests (*a*) both radical formation and substrate activation (Fig. 5)
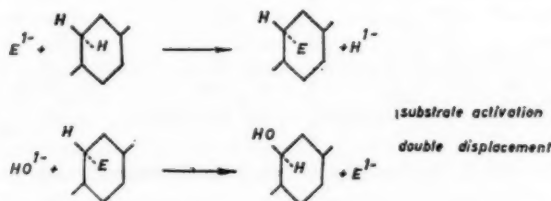
Fig. 5. Steroid hydroxylation (radical formation and substrate activation).

or (*b*) radical formation on the steroid structure (Fig. 6).
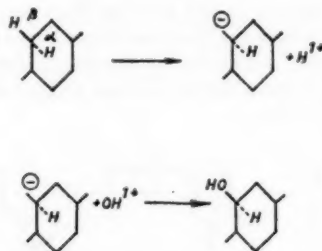
Fig. 6. Steroid hydroxylation (radical formation).

The enzyme in this instance is nondialysable and is heat stable.

AROMATIC RING OPENING

Tryptophane.—The formation of formylkynurenine by oxygenation of tryptophan has been studied by Knox & Tanaka (17) with special reference to the reaction mechanism (Fig. 7).

FIG. 7. Oxygenation of tryptophan.

Catalase interferes with the reaction. This means that small amounts of H_2O_2 are required, and since no evidence could be obtained for Fe^{4+} and Fe^{5+} complexes, the scheme illustrated in Fig. 8 was suggested:
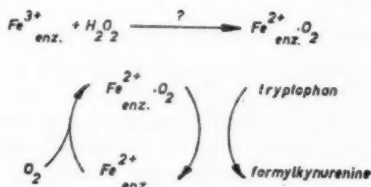
FIG. 8. Enzymatic mechanism for the oxygenation of tryptophan.

The first reaction, namely, the formation of a complex with $Fe_2^+ \cdot O_2$, is an often used hypothesis, but for the findings of Knox, its main interest lies in the useful role H_2O_2 may have in the living cell.

We like to recall that the kynurenine pathway is not the only way found in nature. Mitoma *et al.* (18) have shown that *Chromobacterium violaceum* hydroxylates tryptophan to 5-hydrotryptophan. Apart from the fact that the enzyme is specific, little information is available about the reaction mechanism.

3-hydroxyanthranilic acid oxygenation.—For this reaction Mehler (19) suggests the formation of a labile intermediate, obtained by ring fissure after oxygen addition. Here again Fe^{2+} ions seem to be involved. The primary oxygenated product could be transformed either to picolinic acid or quinolic acid (Fig. 9).
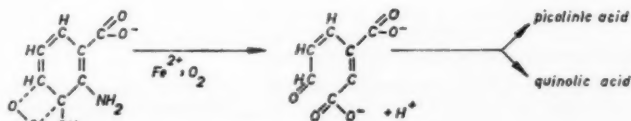
FIG. 9. Oxygenation of 3-hydroxy anthranilic acid.

Ring opening of homogentisic acid.—With homogentisic acid we apparently confront another type of ring opening. Here hydroxylation occurs after dehydrogenation. As homogentisic acid readily changes to the quinone state, this form might be intermediate, as supposed by Lerner (2) (See Fig. 10).
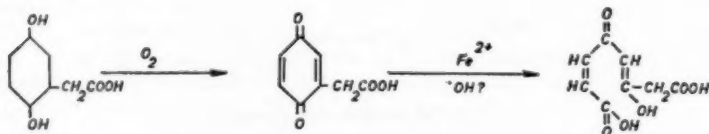
FIG. 10. Oxygenation of homogentisic acid.

The manner in which an iron-enzyme complex could be formed is being studied by Suda (20).

NONSPECIFIC NONENZYMATIC HYDROXYLATION

It is commonly known that a large number of drugs and unnatural aromatic substance are readily metabolized by the intact organism to yield hydroxylated compounds. This means that, apart from the specific enzymatic reactions we have commented on in the paragraphs above, there could be a nonspecific (nonenzymatic) system also. Nonspecific hydroxylation can be brought about by a variety of substances which eventually could be realized in living cell conditions. The study of those model systems may give valuable information on enzymic hydroxylation mechanisms.

The hydroxylating systems are: $H_2O_2 + Fe^{2+}$—or Fe^{3+}—(21) ascorbic acid, metal ions—Fe^{2+}, Fe^{3+}, Cu^{1+}, Co^{2+}—(22 to 25) usually mixed with a complexing agent EDTA + oxygen, and, finally, ultraviolet radiation (26) and ultrasonic radiation (27). Often combinations have been made of the aforementioned systems.

These reagents are all more or less effective in introducing hydroxyl groups in the aromatic nucleus. As far as we know, the stepwise introduction of these groups does not stop at the diphenol stage and may lead to oxidative destruction of the compounds formed.

The mixture of H_2O_2 and Fe^{2+} known as Fenton's reagent, has powerful oxidizing properties studied by Baxendale (28) and others. Free radical formation should be the basic mechanism:

$$Fe^{2+} + H_2O_2 \text{ -- -- -- } \rightarrow Fe^3 + °OH + OH^{1-} \tag{8}$$
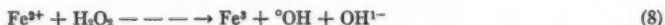
Fe^{2+} is involved in competitive reactions also:

$$\begin{align}
&(a)\ Fe^{2+} + °OH \text{ -- -- -- } \rightarrow Fe^{3+} + OH^{1-} \tag{9}\\
&(b)\ H_2O_2 + °OH \text{ -- -- -- } \rightarrow °HO_2 + H_2O \quad °HO_2 \leftarrow \text{ -- -- } H^{1+} + O_2^{1-}
\end{align}$$

$$\begin{align}
&(a)\ Fe^{2+} + °HO_2 \text{ -- -- -- } \rightarrow Fe^3 + HO_2^{1-} \tag{10}\\
&(b)\ Fe^{3+} + O_2^{1-} \text{ -- -- -- } \rightarrow Fe^{3+} + O_2
\end{align}$$
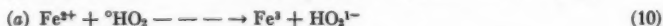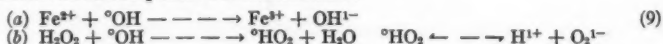
On the basis of the work of Weiss (29), we would like to suggest an additional reaction leading to the formation of a complex ($Fe^{3+} \cdot O_2^{1-}$) which breaks down readily to $Fe^{2+} + O_2$ unless stabilized by anions (F^{1-}, $P_2O_7^{4-}$, OH^{1-}). According to Weiss, this stabilized complex could react with protons:

$$(A^{1-} \cdot Fe^{3+} \cdot O_2^{1-}) + H^{1+} \longrightarrow (Fe^{3+} \cdot A^{1-}) + °HO_2 \qquad (11)$$
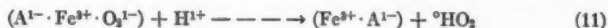
and provide free radicals.

We wonder whether complex stabilization could not be obtained through "substrate" anions. Hydroxylation might then occur as in Fig. 11.
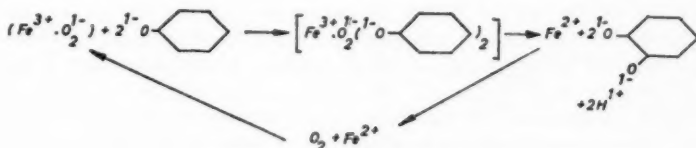
FIG. 11. Scheme for hydroxylation.

This hypothetical scheme applies only to charged substrates. Free radicals could react as in Fig. 12:
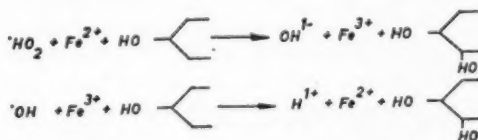
FIG. 12. Hydroxylation of free radicals.

whereas oxidation can be represented as in Fig. 13:
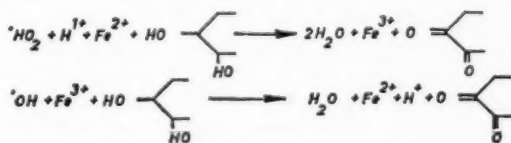
FIG. 13. Oxidation of free radicals.

Baxendale has observed the promotion of reaction (8) by Cu^{2+}. This might explain the good results obtained when fortifying the Fe systems with this metal ion (30).

Reactions (9a) and (10a) explain why the system can work without addition of H_2O_2 (31). This does not mean however that the possibility that H_2O_2 is produced by autoxidation should be neglected.

The actual situation is the result of the relative speed of the different competitive reactions, and we suppose that it will greatly depend upon the chemical structure of the aromatic compound. Ascorbic acid is known to play a role in *in vivo* and *in vitro* hydroxylation. A study of its mechanism of action might make it unnecessary for biochemists to concern themselves further about the "apparent unemployment" of this vital substance. In studies of the hydroxylation of tyramine by adrenal extracts, Udenfriend *et al.* (32) have observed the promoting activity of ascorbic acid. Ascorbic acid + Fe^{2+} (or Fe^{3+}) + EDTA and, to a lesser extent Cu^{1+} or Cu^{2+}, could be substituted for the enzyme extract. In anaerobiosis, addition of as much as 2 moles of H_2O_2 per mole of ascorbic acid could replace oxygen. Diketo- and enedioles could replace ascorbic acid. (D-ascorbic acid, L-isoascorbic acid, dihydroxymaleic acid, ninhydrin, L-dehydroascorbic acid, diketogulonic acid, diethylketosuccinate and alloxan were tested.) On aerobiosis only enediols were active. Udenfriend and his group suppose that a hydroperoxide compound is responsible for the hydroxylation reaction.

The role of ascorbic acid could be that of an intermediate electron acceptor (DeBruyne, personal communication). The experiments of Kersten *et al.* (33) and those of Geyer *et al.* (34) on fatty acid oxidation support this view. They isolated an enzyme system from suprarenal microsomes which oxidizes DPNH in the presence of ascorbic acid and O_2. The system ascorbic acid → mondehydroascorbic acid should form the electron carrier between reduced coenzyme and oxygen. Intermediary hydroxyl radicals probably arise in this way. Stepwise oxidation of ascorbic acid has been observed by Gero & Le Gallic (35). The first step, catalyzed by Cu^{2+}, should result in the formation of a free radical of monodehydroascorbic acid after loss of one H.

In recent studies ethylenediaminetetraacetate has been added as a complexing agent. This substance promotes hydroxylation of model systems as well as oxidations in such isolated biochemical systems as the one studied by Baxter & Van Reen (36).

The oxidation of sulfide is speeded up by the addition of versene, at least for a number of metals (Ni, Fe, Co, Mg) and has either no influence or a retarding influence with Co, Mn, Mo, Zn. Addition of protein (bovine plasma albumin) also favors oxidation. Chelate compounds of Cu (among them folic acid and xanthopterin) enhance the catalytic effect of this metal in melanin formation from dopa [Isaka & Ishida (37)]. Complexing agents and interaction with proteins influence the redox properties of the metals. Discussion of these matters would lead too far from our subject, and we refer the reader to the expert work of Martell & Calvin (38).

In the experiments of Arnow (26) ultraviolet radiation has been shown to produce dopa from tyrosine. Perhaps °OH radicals are responsible. Ultrasonic radiation is also effective [Robert *et al.* (27)]. Robert has revealed the intermediary formation of aromatic radicals by ultraviolet spectroscopy (Fig. 14).
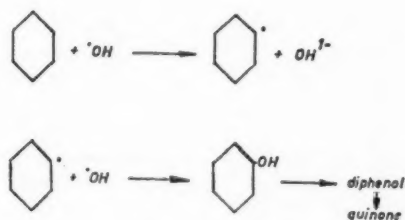
FIG. 14. Intermediary formation of aromatic free radicals.

Another point in nonspecific hydroxylation needs comment: the direction of the hydroxyl group in the aromatic nucleus. *In vivo* [Bray *et al.* (39)] as well as *in vitro* [Brodie (23) and Dalgliesh (24)], the main products are obtained through hydroxylation at electronegative sites. The papers of Dalgliesh (24) and Lissitzky & Roques (25) have shown that this may lead in the case of phenylalanine (β-phenylethylamine and phenylacetic acid) to the removal of the side chain. They suggest the reaction sequences given in Fig. 15.

Lissitzky & Roques (25) have studied especially the hydroxylation of tyrosine, thyronine, and their iodinated derivatives. The hydroxylation at electronegative sites is in line with the hypothesis of free radical formation from the aromatic compounds, resulting from the surplus of one electron after the loss of a proton. This hypothesis has been mentioned already in relation to steroid hydroxylation. A further example of electronegatively directed hydroxylation is found in the next paragraph.

Oxydase and Hydroxylase Activity of Peroxidase

Peroxidase has revealed itself as an enzyme with a very wide spectrum of activities. Mason (40) has shown recently that crystalline horse-radish peroxidase can act as an hydroxylase in the presence of dihydroxyfumaric acid. The mechanism he suggests in his preliminary note is very interesting. He supposes that the Fe of the enzyme should be in the reduced form Fe^{2+}, should complex with oxygen and transfer it as oxyhemoglobin does, without valency changes. The reaction mechanism should be that illustrated in Fig. 16.
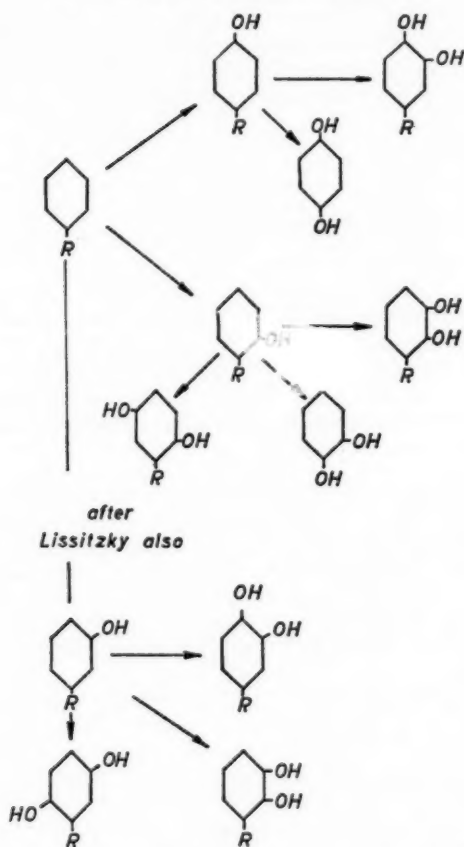
FIG. 15. Hydroxylation at negative electron sites.
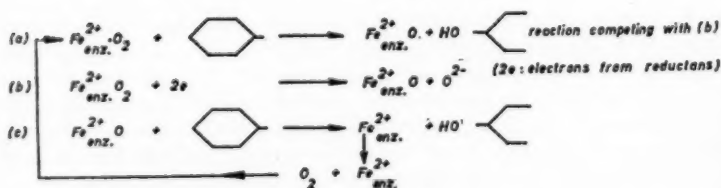
FIG. 16. Mechanism for hydroxylase activity.

This explains why one mole of oxygen is consumed for each mole of dihydroxyfumaric acid. Unlike those given before, this reaction mechanism cannot use ascorbic acid. Cytochrome-c and liver catalase do not give similar reactions. Catalase as well as Mn^{2+} are powerful inhibitors. The OH group is directed toward electronegative sites of the aromatic nucleus.

The proposed participation of iron in the reaction, recalling the hypothesis of Mehler, did not prove to be correct. There is no doubt that iron reacts in another form than Fe^{3+}. Mayrargue-Kodsa *et al.* (22) have studied the action of horse-radish peroxidase on various phenolic amino acids. The main reaction with monophenols is that of polymerisation by removal of hydrogen. Diphenols are oxidized in the presence of H_2O_2 as they were with polyphenoloxidase. Another point was still unexplained by Mason's preliminary note, namely, the inhibition by catalase. He recently (41) suggested that the system he used generates H_2O_2 in two possible ways: (*a*) spontaneous oxidation of DHFA catalyzed by traces of heavy metals. (*b*) enzymatic oxidation by peroxidase working as an oxydase in the presence of Mn^{2+} ions.

$$DHFA + O_2 \longrightarrow H_2O_2 + \text{diketosuccinic acid} \qquad (12$$
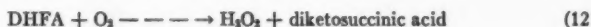
This peroxide can be used in a common peroxidase reaction:

$$H_2O_2 + DHFA \longrightarrow 2\,H_2O + \text{diketosuccinic acid} \qquad (13)$$

or to oxidize Fe^{3+}.

$$H_2O_2 + Fe^{3+} \longrightarrow Fe^{4+} \text{ or } Fe^{5+} \qquad (14)$$
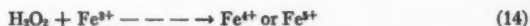
By this transformation peroxidase should show hydroxylase activity. We mentioned before that Knox & Tanaka (17) have described a reaction of Fe^{3+} with H_2O_2 which certainly does not lead to high oxidation levels of the metal. The way Fe is built into the protein (or the prosthetic group) could be decisive.

The oxidase activity of plant peroxidase is a reaction that has been known for a relatively long time. Lemberg & Legge (42) have formulated the reaction in terms which account for increased oxygen uptake, catalase- and CO-inhibition:

$$O_2 + H_2M \longrightarrow H_2O_2 + M$$

$$\underline{Fe^{3+}OH} \diagup + H_2M + H_2O_2 \longrightarrow \underline{Fe^{3+}OOH} \overset{H_2M}{\diagup} + H_2O \qquad (15)$$
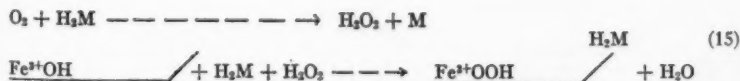
Unlike in normal peroxidase reactions the complex enzyme—H_2O_2—dihydroxymalaic acid breaks down after reduction of iron:

$$
\begin{array}{l}
\underline{Fe^{2+}} \diagup + H_2M + H_2O_2 \\
\quad + O_2 \\
\underline{Fe^{2+} \cdot O_2} \diagup \qquad\qquad \underline{Fe^{2+} \cdot H_2O_2} \overset{H_2M}{\diagup} - - - 2\,H_2O + M \qquad (16) \\
\quad + H_2M \\
\underline{Fe^{2+} \cdot O_2} \overset{H_2M}{\diagup} - - - - - \longrightarrow \underline{Fe^{2+} \cdot H_2O_2} \diagup + M
\end{array}
$$
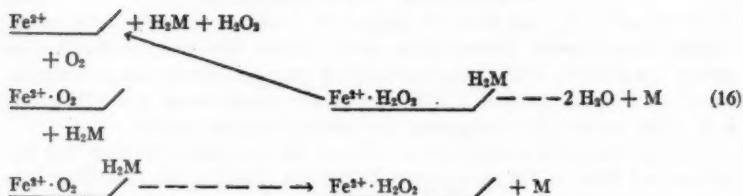
This theory postulates that $Fe^{2+} \cdot O_2$ is responsible for oxidase activity. In this respect, it resembles Mason's original theory for hydroxylase activity.

Yamazaki *et al.* (43) have reported a peculiar oxidase reaction with crystalline turnip peroxidase. The substrate is triose reductone. Dihydroxymaleic acid is less readily oxidized. Ascorbic acid, hydroquinone, catechol, guaiacol, *m*-cresol, and cysteine are not utilized. The oxidase activity is attributable to the peroxidase, since F^- and CN^- are powerful inhibitors. The reaction sequence should be:

(a) $RnH_2 + O_2 - - - - \rightarrow Rn + H_2O_2$ (RnH_2 = triose reductone)
$\quad\quad\quad\quad$ peroxidase $\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad (17)$
(b) $H_2O_2 + RnH_2 - - - - \rightarrow Rn + 2\,H_2O$ (Rn = 2 equivalents in the oxidized form)
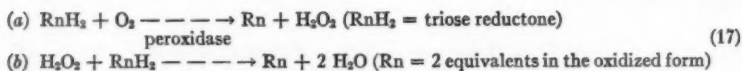
Reaction (b) is the common peroxidase reaction. Reaction (a) is catalysed by Mn^{2+}. The peculiarity of reaction (a) resides in the role of methylene blue. The reaction goes on in anaerobiosis with methylene blue or thionin as a hydrogen acceptor. A trace of H_2O_2 must be added in anaerobiosis. In this condition, Mn^{2+} is ineffective.

$$
RnH_2 + \text{methylene blue} \overset{\text{peroxidase}}{\underset{\text{trace } H_2O_2}{- - - \rightarrow}} Rn + \text{leucomethylene blue} \qquad (18)
$$
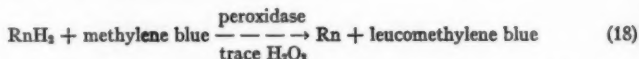
DPNH, phenosafranine, and Nile Blue cannot replace methylene blue.

In our opinion traces of H_2O_2 are required for bringing the Fe into the proper level of oxidation linked with oxidase activity. Intermediate redox systems are known to play a role in the conversion of phenylalanine to tyrosine. This now might be a similar example of oxidase activity displayed by an enzyme which has also hydroxylase properties. The fact that the enzyme catalyzes two different types of reaction is not very surprising. We have met a similar situation in nonenzymatic hydroxylation often followed by oxidation. The missing link is the one between the cofactor as a redox system and the metal ions of the enzyme molecule.

OXYGENATION OF THE STRAIGHT CARBON CHAIN COMPOUNDS

A new avenue is opened by the work of Bloch (44) on the oxygenation of fatty acids followed by the removal of water:

$$
-CH_2-CH_2- + 1/2\,O_2 - \rightarrow \quad -CH_2-CHOH- \rightarrow -CH=CH- + H_2O \qquad (19)
$$

Bloch ascertained that O_2 cannot be replaced by other electron acceptors such as methylene blue, flavine adenine dinucleotide, or ferrocyanide. No further details are available.

Another interesting reaction is that studied by Hayaishi (45). Isotope techniques have shown that L-lactic acid can be directly transformed in acetic acid, CO_2, and water. Lysine was converted by pseudomonas into γ-aminovalerianic acid, NH_3, and Co_2. Anthranilic acid was converted into catechol. No inhibition was observed upon addition of EDTA. Hayaishi stressed the fact that oxygenase activity can account for a fairly high percentage of total oxygen uptake in strictly aerobic bacteria.

It is possible that in time new evidence for staright carbon chain oxygenation will be presented. Besides the uncommon or nonnatural substances we have discussed in the paragraph on peroxidase, we have already a few examples of common metabolites. In time their number may grow and throw some doubt on our present schemes of terminal oxidation.

CONCLUDING REMARKS

If drawing untimely conclusions from the present survey is permitted we might summarize them as follows:

(*a*) A large number of oxygenases and hydroxylases contain a metal, mostly Fe. To display enzymic activity Fe should not be in the Fe^{3+} state. A higher oxidation level (Fe^{4+} or Fe^{5+}) or complexes with oxygen (even with peroxide?) as, for example, $Fe^{2+} \cdot O_2$ or may be $Fe^{3+} \cdot O^-_2$ are required. These complexes may arise by reaction with H_2O_2 or with oxygen. These iron compounds mediate either the formation of °OH radicals or transfer oxygen to the substrate. Free radical formation on the substrate may be the reason hydroxylation is directed toward electronegative sites. Sometimes there is a preliminary dehydrogenation. Intermediate redox systems, both organic and metallic, have been discovered. Chelating agents and enzyme proteins favour the reaction and should be responsible for specificity.

(*b*) The study of the reaction mechanisms has opened a new avenue for the interpretation of a number of biochemical problems. The discovery of intermediate redox systems reacting either with DPNH or TPNH strongly supports the idea that oxygenase and hydroxylase activity is a route for terminal oxidations. The biological role of peroxidase and possibly that of catalase also can be explained now by its oxidase and hydroxylase activity. Traces of H_2O_2, suspected to arise during respiration, can be consumed in useful reactions preparing the metal ion for oxygenase activity.

At first glance, direct oxygenation and hydroxylation may appear as a waste of energy, since no trapping device has been found. These reactions should be seen as sparking reactions which invariably lead to degradation products which are common cellular metabolites. Transiently powerful hormones may be formed.

(*c*) The question whether nonenzymic, nonspecific hydroxylation is im-

portant cannot be solved at present. Fellman & Delvin (46) believe that beef adrenal medulla hydroxylate phenylalanine by a mechanism suggested by Udenfriend. So does Dennell, as a result of his study on insect cuticle hydroxylation (47).

Comparative kinetic studies could discover to what extent nonspecific, nonenzymic hydroxylating systems are able to compete with the enzymatic reaction. If for genetic or other reasons the normal enzymatic pathway is blocked, nonenzymic systems might be very important.

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METABOLISM OF CONNECTIVE TISSUE[1,2]

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This review will stress the metabolism of certain carbohydrate consti-
tuents of connective tissue and related compounds. Only a few of the
numerous publications in this field will be reviewed, because of limitations
of space.[4]

CONNECTIVE TISSUE

There is apparently no exact definition of connective tissue (12, 13, 14).
Robb-Smith regards connective tissue as "a continuous fluid matrix varying
in consistency from the limpid Wharton's Jelly of the umbilical cord to the
hardness of bone, in which is lying an interlacing fabric of fibers of differ-
ent sorts . . ." (13). Thus, connective tissues from different areas of the
body vary considerably in chemical composition, despite the fact that the
tissue is regarded as "continuous." The composition of the tissue in certain
areas also changes with age (15).

Important physiological phenomena, such as the aging process, wound
healing, certain immunological reactions, "connective tissue diseases," calcifi-
cation and bone resorption, apparently depend in large measure on the state
of the connective tissue and the cells which produce the tissue. An under-
standing of these processes will probably not be realized until we have
detailed information on the special metabolic functions of connective tissue

cells, changes in these functions with age, metabolic differences between cells which cannot be distinguished by visual techniques, and finally the control mechanisms for these metabolic reactions (which may be hormonal, nutritional, etc.).

The cells of connective tissue consist of the following types: fibroblasts, undifferentiated cells, macrophages (histiocytes), mast cells, and a much smaller number of other types. The fibroblasts, the most common cell type, have been grown in tissue culture and are apparently responsible for formation of the intercellular cement substance. The claim that the mast cells are responsible for the production of hyaluronic acid (14) is not generally accepted (16), although heparin as well as hydroxytryptamine and histamine are reported to be present in mast cell granules (17).

A large portion of connective tissue is extracellular material. It consists of fibrous proteins such as collagen and of amorphous material called "ground substance" or intercellular cement substance which lies around and between the various fibers and cells. The ground substance is a complex mixture containing tissue fluid (originating from the plasma), various metabolites produced by the cells, the so-called "mucoid substances," lipides, and proteins. Studies on the nonfibrous proteins indicate the variability of connective tissue. Rat subcutaneous tissue contains substantial amounts of proteins with electrophoretic mobility similar to serum proteins (18). These observations have been extended (19); plasma proteins are present in the skin and tendon of the rat, rabbit, and ox. The total quantity of plasma protein present in rabbit skin is equal to 25 to 30 per cent of the total protein in the plasma. Fractionation of the skin albumin fraction yields plasma albumin, and also gives two other proteins which resemble serum albumin but are not identical with it. Cartilage, on the other hand, contains noncollagenous protein which is not thought to be plasma protein (20). The free pore spaces of the cartilage gel are of the order of 10 to 15 A (21, 22), which would allow the penetration of cartilage by simple sugars, amino acids, etc., but not by large molecules. If this concept is correct, it is difficult to understand how the high molecular weight constituents of cartilage (e.g., collagen, chondromucoprotein,) can be synthesized and laid down in the cartilage matrix except in the area immediately surrounding the cells.

While some of the constituents of the ground substance have been isolated and characterized, relatively little is known about the interrelationships between these substances. The physiological importance of such interdependence was dramatically illustrated by McCluskey & Thomas (23) and by Bryant et al., (24). Intravenous injection of papain into rabbits resulted in loss of chondroitin sulfate from cartilage matrix throughout the body; the matrix was restored within several days. The available evidence suggests that the papain effect results from proteolysis rather than from the hydrolysis of the polysaccharide. In view of these results, experiments which lead to variations in the chemical composition of connective tissue, "turnover

rates" of isolated components such as polysaccharides, etc., should be interpreted with caution since such effects may be indirect.

MUCOID SUBSTANCES

The variable nature of the ground substance suggests the difficulty in obtaining reliable chemical descriptions of this material. Most workers agree, however, that the ground substance contains considerable quantities of the "mucoid" substances, i.e., carbohydrate-containing materials of high molecular weight. Chemical information concerning the nature of the mucoid substances is so limited that numerous systems of nomenclature have appeared (25), and none of them is generally accepted. For this review the nomenclature indicated in Table I will be used.

TABLE I

NOMENCLATURE*

Per Cent Carbohydrate	Names	Examples
0	Protein	Insulin
Trace to 15	Glycoprotein, Protein	Albumins, Globulins, Collagen
10 to 85	Mucoproteins	Orosomucoid, Blood group substances, Urinary mucoprotein
65 to 100	Mucopolysaccharides	Hyaluronic acid, Chondroitin sulfates, Chitin, Heparin
100	Polysaccharides	Cellulose, Hemicelluloses

* Only high molecular weight compounds which contain carbohydrate and/or amino acid are considered. (a) The compounds are classified on the basis of their relative quantities of amino acid and carbohydrate. However, it should be stressed that there are no sharp lines of demarcation; the groups therefore overlap, and the values used for classification are strictly arbitrary numbers which will probably change as more precise information is gathered. (b) Few proteins are carbohydrate-free. (c) While the mucopolysaccharides can be isolated as high molecular weight substances free of protein or peptide residues, separation of amino acids from carbohydrate residues in the glyco- and mucoprotein classes requires extensive degradation to low molecular weight compounds. However, the mucopolysaccharides may be combined with proteins *in vivo*. As indicated elsewhere in this volume, chondroitin sulfate of cartilage exists in complex with protein, although the nature of the bond is unknown. In other cases, notably hyaluronic acid and heparin, it is not yet clear whether the polysaccharides exist in complex with protein by salt or hydrogen bonding or by some labile covalent bond. (d) The present concept of glyco- and mucoprotein structure suggests that these substances contain oligosaccharides and peptides which are covalently bonded to yield high molecular weight compounds. The nature of these bonds is reviewed elsewhere in this volume. (e) The difference between the glyco- and mucoproteins resides in their chemical and physical properties. Thus, the mucoproteins generally have a higher carbohydrate content and are more stable to a variety of agents such as heat, protein precipitants, etc. (11). (f) The carbohydrate constituents of the substances in this table vary considerably, but, with few exceptions, the "mucoid" substances contain hexosamine.

Certain "mucoid" substances also contain lipides, but the situation here is even more confusing than that described above. A comprehensive review on brain lipides [Lebaron, F. N., and Folch, J., *Physiol. Revs.* 37, 539 (1957)] indicates the complexity of the problem. Rosenberg & Chargaff [*J. Biol. Chem.*, 232, 1031 (1958)] have recently proposed a definition for mucolipides; they are "soluble in water, but also in organic solvents, contain fatty acid, a sphingosine-like base, a hexose, also amino sugar, sometimes amino acid, and most significantly, sialic acid or a related substance." It should be noted these substances would differ from many of the lipopolysaccharides [Stacey, M., *Advances in Carbohydrate Chem.*, 2, 162 (1946)].

The tissue sources for some of the mucopolysaccharides are shown in Table II. These data are derived from the work of Meyer *et al.* (26), who characterized the polysaccharides after isolation. The number of polysaccharides in connective tissue remains unknown, although the work of Meyer and his colleagues showed the presence of at least six: hyaluronic acid, chondroitin sulfates A, B, and C, keratosulfate, and chondroitin. Other known mucopolysaccharides, such as heparin, heparitin sulfate (27),[5] etc., are not included in Table II, although there is some information on the distribution of these compounds. An understanding of connective tissue biochemistry will be possible only by the separation, isolation, and characterization of its components. Ultimately, histochemical techniques will provide more accurate information, but interpretations of the histochemical data

TABLE II

DISTRIBUTION OF SOME MUCOPOLYSACCHARIDES[*]

Tissue	Hyaluronic Acid	Chondroitin sulfate			Keratosulfate	Chondroitin	Others
		A	C	B			
Vitreous Humor	+						
Synovial Fluid	+						
Fowl Tumors	+						
Liposarcoma	+						
Cartilage		+	+	+			
Adult Bone		+					
Chondrosarcoma		+	+				
Chordoma			+				
Umbilical Cord	+		+				
Fibroblasts (tissue culture)	+		+				
Electric Eel	+		+				
Pig Skin	+			+			
Ligamentum Nuchae	+	+		+			
Tendon	+		+	+			
Heart Valve	+		+	+			
Cornea		+			+	+	
Calf Bone		+	+		+		+
Aorta	+	+		+			+

* See Meyer *et al.* (26) for quantitative relationships. This table is not intended to be complete. For example, HA occurs in tissue and tissue exudates other than those indicated (e.g., human mesothelioma). Further, other mucopolysaccharides, such as heparin, occur in some of the tissues referred to above. Minor components may be present in all tissues, but would not be detected by the fractionation procedures.

[5] There is no generally accepted nomenclature for the various mucopolysaccharides. For example, chondroitin sulfate B has also been designated β-heparin, dermoitin sulfuric acid, and gastroitin sulfuric acid. The nomenclature problem is fully reviewed elsewhere in this volume by Pigman *et al.*

presently available must be made with extreme caution. Promising developments in histochemical methodology are the discovery of an increasing number of enzymes which can hydrolyze certain of the mucopolysaccharides. The isolation of purified, specific enzymes would be of considerable aid; information obtained with crude enzymes can be misleading (28).

The analysis of connective tissue is complicated by the fact that the homogeneity of an isolated fraction is difficult to determine. The history of chondroitin sulfate illustrates this point; initially, only one was recognized (29), then three fractions were isolated (26), and now it is suggested that chondroitin sulfate B consists of two fractions (30). The development of new procedures for the separation of mucopolysaccharides is therefore of great importance. These include the use of paper chromatography (31), paper electrophoresis (32, 33), and column chromatography (34). Two methods appear to be of particular interest. Polyanions can be precipitated quantitatively with quarternary amines (35); precipitation is a function of the charge of the polyanion, the concentration of salt, etc. The method has already been applied to the fractionation of heparin (36) and promises to be one of the most useful techniques in this field. Another method which may have wide application is electrophoresis of polysaccharides on glass-fiber paper in alkali (37), which resulted in the separation of polysaccharides like glycogen into two or more components. This study showed that a number of polysaccharides, heretofore believed to be the same, were different and that others which were considered to be homogeneous, were not.

Recent work on the chemistry and isolation of several mucopolysaccharides is reviewed elsewhere in this volume by Pigman *et al.* Some of the many recent studies on the analysis of connective tissue will be mentioned here. The change in composition of pig skin from embryo to adult stages was studied (38), and considerable differences were found in the relative concentrations of chondroitin sulfates B and C and hyaluronic acid. Linker *et al.* (27) reported the distribution of heparitin sulfate. The nature and nomenclature of the chondroitin sulfates was discussed (39). A number of laboratories have reported the presence of mucopolysaccharides in leukocytes, blood platelets, urine, plasma, etc., and these studies are reported in the paper by Kerby (32). Hyaluronic acid is reported as a constituent of Heberden's nodes (40) and sulfated polysaccharides in bovine cornea and sclera (41). An unidentified polysaccharide which contains hexosamine and hexuronic acid is reported to be present in the myelin sheath (42). A provocative but preliminary report by Hall *et al.* (43) suggests the presence of cellulose fibers in mammalian tissue. While cellulose does occur in tunicata, this polysaccharide has not previously been found in higher animals. If the findings by Hall *et al.* are confirmed, they may have widespread biological implications in the field of connective tissue physiology.

The monosaccharides isolated from connective tissue mucoids are: D-galactose, D-mannose, L-fucose, D-glucuronic acid, L-iduronic acid, D-glucosamine, D-galactosamine, and the sialic acids. D-glucose occurs only infre-

quently. The hexosamine moiety of the sialic acid appears to be D-mannos-amine. In the case of the bacterial mucoid substances, L-rhamnose frequently occurs, while L-fucose occurs relatively infrequently. The structural relation-ship of D-glucuronic and L-iduronic acid is shown in Figure 1, along with L-guluronic and D-mannuronic acids. The latter two acids are components of the seaweed alginic acids (44). Possibly L-iduronic acid arises from D-glucuronic acid and L-guluronic arises from D-mannuronic acid by epimeriza-tion at C-5.
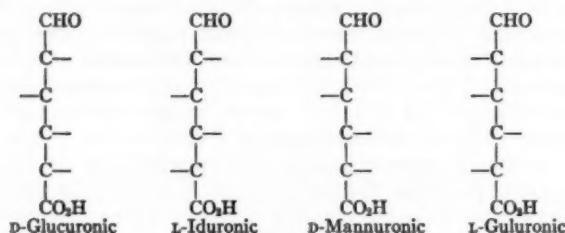
Fig. 1. Some natural uronic acids.

Unfortunately, the carbohydrate substituents have frequently been char-acterized only by colorimetric and chromatographic techniques. For example, L-fucose has rarely been isolated and characterized as the L-isomer, despite the fact that the D-isomer is known to exist in nature (25). The isolation of an increasing number of uronic acids, deoxy sugars (45), amino sugars such as talosamine (46), etc., suggests the need for adequate characteriza-tion. In this respect, the isolation of an aldoheptose (47) and an aminohexu-ronic acid (48) are of interest. Reliance on colorimetry and paper chromatog-raphy led to misinterpretations concerning the structure of the sialic acids and the metabolism of galactosamine.

MONOSACCHARIDE METABOLISM

Sugar nucleotides.—Recent information indicates that the sugar nucleo-tides are of prime importance in connective tissue carbohydrate metabolism. These substances were discovered independently by two groups (49, 50). The exciting findings of Leloir and his colleagues (51) present major new approaches to many problems of carbohydrate metabolism.

The sugar nucleotides are of the general formula indicated below:

Base = Purine or pyrimidine
Glycose = Sugar residue
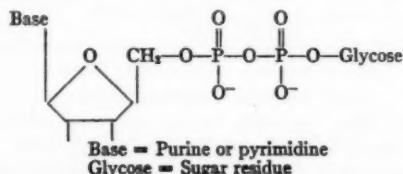
Where the nucleoside is guanosine, two compounds are known; guanosine diphosphate mannose (52) (GDP-mannose) and the corresponding fucose, presumably L-fucose, derivative (53, 54) (GDP-fucose). The list of uridine sugar nucleotides continuously increases. It includes UDP derivatives of the following sugars; N-acetyl-D-glucosamine N-acetyl-D-galactosamine, D-glucosamine, D-glucose, D-galactose D-glucuronic acid, D-galacturonic acid, D-xylose, L-arabinose, muramic acid, and muramic acid peptides. A phosphate ester of UDP-acetylglucosamine and a sulfate ester of UDP-acetylgalactosamine have been reported (55). The isolation of cytidine diphosphate ribitol and glycerol are also of interest. The nucleotides are the subject of a recent review (56) and are discussed below in reference to their metabolism.

The isolation and characterization of individual nucleotides is a difficult problem which has not yet been satisfactorily resolved. Generally, the procedures require extraction of the tissue, deproteinization, fractionation of the mixed nucleotides by ion exchange chromatography, adsorption, and elution of the nucleotides from charcoal. In certain cases, such as UDP-acetylgalactosamine (57) and GDP-fucose (54), the nucleotides were not separated from similar compounds. The studies of Denamur et al. (58) on the nucleotide composition of sheep milk indicate the importance of the methods used for deproteinization and of the temperature maintained during isolation. GDP-fucose was almost absent when deproteinization and fractionation were carried out under the usual conditions. Similarly, UDP-glucuronic acid has long been known to be much less stable than compounds like UDP-glucose. The maintenance of low temperatures during ion exchange chromatography was also emphasized (59) in studies on the acid-soluble nucleotides of salmon liver. UDP was not detected and the appearance of UDP in ion exchange chromatography was suggested to be an indication of the decomposition of labile UDP-glycose compounds. In regard to the analysis of nucleotide peaks, it may be noted that sugar phosphates as well as nucleotides are adsorbed by charcoal under certain conditions (60). The adsorption by charcoal of nucleotide and sugar from a solution containing these substances, followed by subsequent elution, is therefore not necessarily evidence that they exist in the same molecule. Improved methods for separation of some of the nucleotide diphosphate sugars have been reported (61, 62). An interesting procedure for such separations involves the use of paper curtain electrophoresis (63), which gives separations at a pH close to neutrality.

Sugar nucleotides are present in all cells which have been examined, although, as suggested above, it appears unlikely that quantitative and even qualitative relationships of the different nucleotides from a single source have been accurately established. In many cases, unidentified nucleotide peaks were isolated by ion exchange chromatography. Sheep milk appears to be a particularly rich source of sugar nucleotides (58, 54); most of the nonprotein organic phosphate in the milk is of this type. A nucleotide-con-

taining amino sugar was isolated from crabs (64); the compound was reported to be different from UDP-acetylglucosamine in respect to the hexosamine moiety. A number of unresolved uridine nucleotides were obtained from a species of group A streptococcus which produces hyaluronic acid (65). A guanosine nucleotide has been isolated from brewer's yeast and was not present in baker's yeast; the nucleotide had the surprising composition guanosine monophosphate-X. The phosphate was shown to be attached to the 3′ position of the ribose moiety (66). A preliminary communication (67) reported the isolation of sialic acid-containing uridine nucleotides from *Escherichia coli*-K235.

In view of the problems of separation and the small quantities available, it is not surprising to note that relatively few of the sugar nucleotides have been completely characterized. The synthesis of UDP-glucose in good yield by Moffatt & Khorana (68) points the way to confirmation of the presumed structure of the natural products.[6] It should be stressed that in many of the reports in the literature, the sugars are inadequately characterized, the D- or L-configuration is frequently assumed, as is the pyranose ring structure and the α-configuration of the glycosidic bond. Presumptive evidence suggests that all the naturally occurring sugar nucleotides are of the α-configuration with the exception of L-fucose and L-arabinose. This problem has not received sufficient attention, particularly since the nucleotides can serve as glycosyl donors in the formation of polysaccharides, which may be either α- or the β-configuration. For example, while β-glucose 1-P is rare, it does occur (69), and, by inference, the β-isomer of UDP-glucose may also exist.

Biosynthesis of sugar nucleotides.—The enzymatic formation of the sugar nucleotides indicated above can proceed by one of the following mechanisms:

$$U \text{ (or G)}—R—P—P—P + X—1—P \rightleftharpoons U \text{ (or G)}—R—P—P—X + P—P \qquad (1)$$
$$U \text{ (or G)}—R—P—P—X \rightleftharpoons U \text{ (or G)}—R—P—P—Y \qquad (2)$$
$$UR—P—P—X + Y—1—P \rightleftharpoons UR—P—P—Y + X—1—P \qquad (3)$$
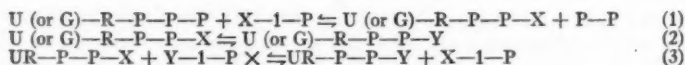
FIG. 2. Synthesis of sugar nucleotides. G = D-guanine; U = uracil; X and Y = glycose residues

In Reaction 1, a sugar phosphate such as α-glucose-1-P and UTP are converted to UDP-glucose plus P-P by enzymes which are called pyrophosphorylases. In Reaction 2, a sugar nucleotide is converted to a different sugar nucleotide by enzymes which catalyze one or more of the following reactions on the sugar residue X to yield Y; oxidation, reduction, epimerization, or degradation by removal of a molecule of carbon dioxide (from UDP-hexuronic acid). Of course, epimerization reactions occur not only with sugar nucleotides but also with free sugars, sugar phosphates (70), and

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[6] The synthetic procedure for UDP-glucose has been applied to the preparation of UDP-glucuronic acid, GDP-mannose (Roseman, Moffatt, Khorana, unpublished work) and to UDP-acetylglucosamine (Maley, unpublished work).

by inversion of the sugar chain as in the case of L-xylulose $\rightleftharpoons$ D-xylulose (71). Reaction 3 has been described only in the case of Gal-1-P, where there is a transfer of the UDP from UDP-glucose to the galactose-1-P with formation of UDP-galactose.

Metabolism of the aldhohexoses and the 6-deoxyaldohexoses.—Figure 3 illustrates the know metabolic pathways starting with glucose-6-P, galactose, and mannose, and leading to the nucleotides containing these sugars and L-fucose. The interconversion of mannose-6-P, and fructose-6-P (Reaction 5) was studied with extracts of pig red blood cells (72), and it was sug-
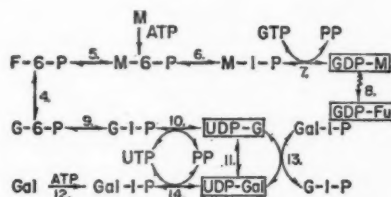
Fig. 3. Galactose, glucose, mannose, and fucose metabolism. (F = fructose, Fu = fucose, G = glucose, Gal = galactose, GDP = guanosine diphosphate, GTP = guanosine triphosphate, M = mannose)

gested that this enzyme may be different from the one previously reported in rabbit muscle (73). An enzyme preparation obtained from *Pseudomonas saccharophila* (74) interconverted a number of nonphosphorylated aldoses and ketoses including mannose $\rightleftharpoons$ fructose but was inactive with D-glucose. Phosphoglucose isomerase (Reaction 4) was not obtained in a purified form prior to its recent isolation from human erythrocyctes (75).

UDP-glucose pyrophosphorylase (Reaction 10) occurs in animal, bacterial, and plant cells; it was first demonstrated (76, 77) in yeast. Crude mung bean extracts (78) catalyze pyrophosphorolysis of a number of uridine nucleotides (Reaction 1). The purified UDP-glucose pyrophosphorylase from the mung bean extract was specific for UDP-glucose (79); the pea seed (80) enzyme exhibited an equilibrium constant of 7.2 for the reaction

$$K = \frac{UTP \times Glucose{-}1{-}P}{UDP{-}Glucose \times PP}.$$

A preliminary report (81) indicated that the pyrophosphorylases from yeast which act on UDP-acetylglucosamine, UDP-glucose, and GDP-mannose were separated by means of DEAE-cellulose column chromatography. A 3000-fold purification of the UDP-glucose pyrophosphorylase was obtained.

The metabolism of galactose has been reviewed (82, 83). These studies,

encompassing Reactions 10 through 14 explain the enzymatic defect in galactosemia and indicate the probable mechanism for the conversion of UDP-glucose to UDP-galactose (Reaction 11). As demonstrated by Kalckar and his co-workers, the defect in galactosemia is the lack of the enzyme galactose-1-P uridyltransferase which catalyzes Reaction 13. This reaction appears to be the major route of utilization of galactose-1-P in the mammal. Reaction 14 had been previously reported in yeast (84) and in plant extracts (78) but not in mammals. While the galactosemic individual shows an inability to metabolize galactose, these patients develop an increased ability to utilize ingested galactose with increasing age. This phenomenon has recently been explained by Isselbacher (85) who showed that the galactosemic individual develops UDP-galactose pyrophosphorylase activity and is therefore capable of handling galactose-1-P via Reaction 14.

Galactose metabolism in *P. saccharophila* proceeds (86): D-galactose $\longrightarrow$ D-galactono-γ-lactone $\longrightarrow$ D-galactonic acid $\longrightarrow$ 2-keto-3-deoxygalactonic acid $\longrightarrow$ pyruvic acid plus D-glyceraldehyde-3-phosphate. Similar pathways exist in this organism for the oxidation of D-glucose, L- and D-arabinose. These enzymes have not yet been demonstrated in animal tissues.

Within recent years a number of laboratories have become interested in the metabolism of the 6-deoxyaldohexoses. Bacterial systems appear to be more convenient for metabolic studies with these sugars than are mammalian tissues. Many bacteria produce polysaccharides or lipopolysaccharides containing L-rhamnose (6-deoxy-L-mannose); several strains synthesize polysaccharides containing L-fucose (6-deoxy-L-galactose) (87). The biosynthesis of L-rhamnose was studied in *Pseudomonas aeruginosa* (88, 89) utilizing whole cells and a variety of labeled compounds. The data suggested either a direct conversion of the fructose carbon chain to that of L-rhamnose or fragmentation to trioses and recombination. Isotope studies on the biosynthesis of L-fucose yielded data which were more readily interpretable since randomization of the C^{14} was minimal. The bacterial systems used in these studies produced polysaccharides containing D-glucose as well as L-fucose. Preliminary reports (90, 91, 92) indicated that the isotope distribution of the L-fucose paralleled that of the D-glucose in the polysaccharide, although in the latter studies there was a significant degree of randomization of the C^{14} when either G-1-C^{14} or G-6-C^{14} was used in the growth medium. The isotope distribution in the D-glucose, D-galactose, and L-fucose components of the bacterial polysaccharide (93) showed no significant randomization when glucose-1-C^{14} or glucose-6-C^{14} was the carbon source, and the isolated monosaccharide constituents of the polysaccharide corresponded in labeling patterns with the glucose used in the medium. On this basis, the carbon skeleton of L-fucose was thought to originate from D-glucose without cleavage or inversion of the carbon chain. More detailed and precise information on the origins of L-fucose has recently been discovered by *in vitro* experiments. Following the isolation of GDP-fucose from sheep milk (54) and from *Aerobacter aerogenes* (53), Ginsburg (94) reported the con-

version of GDP-mannose to GDP-fucose in the presence of extracts from *A. aerogenes.* The system required TPNH. Since the over-all conversion of GDP-mannose to GDP-fucose involves epimerizations at C-3,-4, and -5 and reduction at C-6, Ginsburg concluded that the reaction probably involves several steps. This report represents the first example of a reductive step at the sugar nucleotide level.

The dissimilation of the 6-deoxyhexoses by bacterial systems has also been investigated. Bacteria can grow on L-fucose or L-rhamnose as the sole carbon source. Apparently, the first step in the utilization of these sugars is the conversion of the 6-deoxyaldose to the corresponding 6-deoxyketose by isomerases (95 to 98). The further metabolism of the 6-deoxyketoses apparently involves phosphorylation. Thus, extracts of bacterial cells phosphorylated L-rhamnulose (97, 99); the position of the phosphate group was not established, but was suggested to be at C-1. In the case of L-fuculose, a kinase was obtained, free of isomerase, which phosphorylated L-fuculose but not L-fucose (100). The phosphate ester was isolated and characterized as L-fuculose-1-P by periodate oxidation studies. The further metabolism of L-fuculose-1-P by crude extracts was reported to yield triose fragments.

Uronic acid and pentose metabolism.—Within recent years, a large body of information has accumulated regarding the metabolism of the uronic acids and pentoses. A recent review by Utter (71) summarizes this work; some of the subsequent publications will be discussed below. Most of the work to be discussed is published in the form of preliminary communications, therefore the pathways of metabolism presented in Figures 4 and 5 may require considerable revision as more precise information is obtained.
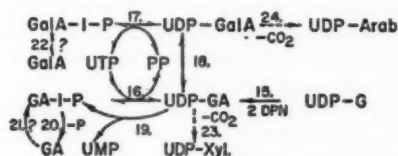
FIG. 4. Glucuronic and galacturonic acid metabolism. (Arab = arabinose, G = glucose, GA = glucuronic acid, GalA = galacturonic acid; Xyl = xylose)

The two major pathways of uronic acid metabolism involve the phosphorylated derivatives shown in Figure 4, or the free sugars shown in Figure 5. Reaction 15, the conversion of UDP-glucose to UDP-glucuronic acid, is well established and occurs in animal, plant, and bacterial cells (101, 102); it is apparently irreversible. UDP-glucuronic acid can also be formed from glucuronic acid-1-P and UTP (Reaction 16), although this enzyme has been found in plant extracts but has not been detected in extracts from animal tissues (101). The physiological significance of Reaction 16 in plant tissues remains undetermined. Thus it may serve as a source for D-glucuronic acid
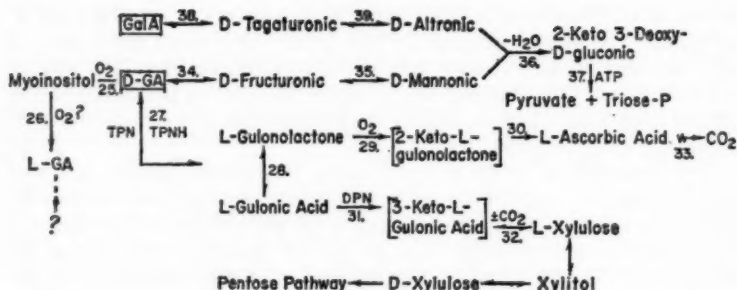
Fig. 5. Glucuronic and galacturonic acid metabolism. (GA = glucuronic acid, GalA = galacturonic acid)

(Reaction 20) which can be utilized for pentose formation, etc., or it may be an important pathway for the formation of UDP-glucuronic acid. In the latter case, the synthesis of D-glucuronic acid-1-P must be considered; neither a kinase (Reaction 21) nor a dehydrogenase which will act on glucose-1-P to yield glucuronic acid-1-P has been described. D-glucuronic acid is formed by hydrolysis of UDP-glucuronic acid (Reaction 19 plus 20) by fractions obtained from rat kidney (103).

Rat kidney extracts convert myoinositol to a racemic mixture of D- and L-glucuronic acids (104). Subsequently (105, 106), the enzyme systems responsible for this oxidation were separated and the one which produced the D-isomer was purified; the oxidation of inositol apparently occurs between C-1 and C-6. The mechanism of formation of L-glucuronic acid is not yet known, but it may proceed either by oxidation of the inositol ring between C-3 and C-4, or by epimerization of the D-glucuronic acid to L-glucuronic acid. In any case, it is indeed surprising that both isomers are produced in equal quantity by crude extracts, since this indicates that both enzyme systems act at equal rates. As discussed below, D-glucuronic acid is metabolized very rapidly by animal, plant, and bacterial cells. On the other hand, nothing is known about the further metabolism of L-glucuronic acid. The fact that this compound is obtained from myoinositol at the same rate as the D-isomer suggests its importance.

The pathway outlined in Figure 5 for the conversion of D-glucuronic acid to L-ascorbic acid and L-xylulose indicates the present thinking of one group of investigators in this field.[7] Previously, Bublitz et al. (107) presented data suggesting the participation of 3-keto-L-gulonate in the formation of both L-ascorbate and L-xylulose, although the keto acid was not isolated. Subsequently, Ashwell et al. (108) reported the isolation of the 3-keto compound in the L-xylulose system. Using a rat liver microsome preparation,

[7] Dr. Gilbert Ashwell, private communication.

the same workers (109) presented data implicating 2-keto-L-gulonic acid in the conversion of L-gulonolactone to L-ascorbic acid. If these formulations are correct, the lactones are involved in the formation of L-ascorbic acid, while the free acids participate in the formation of pentose. The inter-conversion of L-gulonolactone and L-gulonic acid (Reaction 28) was catalyzed by an aldonolactonase (110), which exhibited a broad specificity, although a glucuronolactonase described by the same authors was much more specific.

The dissimilation of L-ascorbic acid is known to produce CO_2 (Reaction 33). There appears to be little information or agreement about the pathway (111 to 114) although dehydroascorbic acid and diketogulonic acid are probable intermediates. In view of the profound effect of L-ascorbic acid on connective tissue, further developments in these areas should prove most interesting.

Although the reactions are not indicated in Figure 5, D-galacturonic acid is as effective a precursor of L-ascorbic acid as D-glucuronic acid. These data are cited in the references indicated above. The isotope data support the enzymatic results outlined in Figure 4. Preliminary communications (115, 116) indicate that bacterial and plant extracts contain an epimerase which catalyzes the conversion UDP-glucuronic acid to UDP-galacturonic acid (Reaction 18). UDP-galacturonic acid can also arise by the pyrophosphorylase Reaction 17, and this enzyme is different from the UDP-glucose pyrophosphorylase (79). The conversion of D-glucuronic acid to D-galacturonic acid as shown in the radioactive studies suggests the possibility of a kinase (Reaction 21). Neufeld et al. (116) have not only reported the epimerization of UDP-glucuronic acid to UDP-galacturonic acid with plant extracts, but also the important observation that these extracts produce UDP- arabinose (Reaction 24) and UDP-xylose (Reaction 23), presumably by decarboxylation of the uronic acid derivatives. The authors note that final characterization of the sugars is incomplete. Assuming that the reactions proceed as indicated in Figure 4, then arabinose would be of the L- and xylose of the D-configurations. These observations present a new pathway for pentose formation, confirming an old idea that pentoses originate in plants by the conversion of hexose to hexuronic acid to pentose by loss of the C-6 as CO_2. The mechanisms for Reactions 23 and 24 have not been established.

The further metabolism of the D-glucuronic acid and D-galacturonic acid in bacteria apparently proceeds along somewhat different lines than in animal tissues. Preliminary communications from three laboratories (117, 118, 119) agree that the first reaction is an isomerization (Reactions 34, 38) which converts alduronic acids to the corresponding keturonic acids. The keto group is then reduced by DPNH or TPNH; different products were obtained with different systems. Thus, Ashwell et al. (120) used extracts obtained from E. coli (ATCC no. 9637) which were grown on the uronic acids and reported Reactions 35 and 39. On the other hand, Payne & McRorie (119) and Kilgore & Starr (118) report that extracts of Erwinia

carotovora or *Aerobacter cloacae* reduced the keturonic acids in the presence of TPNH much more rapidly than DPNH, and that the products were the corresponding L-onic acids (the newly formed hydroxyl group on C-2 is on the right of the carbon chain) which were identified by paper chromatography. The *E. coli* extracts further metabolized D-altronic and D-mannonic acids via Reaction 36 to 2-keto-3-deoxy-D-gluconic acid which finally, in the presence of ATP, was converted to pyruvate and triosephosphate (120). The 2-keto-3-deoxy type of compound and its further metabolism (Reaction 37) to trioses fits into the pathway of hexose metabolism previously established by Doudoroff and his associates (86, 121, 122) and Kovachevich & Wood (123). Further, as discussed below, this pathway is apparently involved in D-glucosaminic acid metabolism.

With regard to the interconversion of the sugar acids, a recent communication (124) reports the presence of a lactone epimerase which reversibly converted D-glucono-δ-lactone to D-mannono-δ-lactone; the enzyme was apparently quite specific. This type of epimerase may play important roles in some of the reactions discussed above.

Linker *et al.* (125) have studied a unique type of glycosidase, i.e., the bacterial hyaluronidases. These enzymes hydrolyze hyaluronic acid to the disaccharide shown in Figure 6; apparently water in the medium is not utilized, but the "hydrolysis" occurs by cleavage of the glucosaminidic bond utilizing the hydrogen at the C-5 position of the uronic acid moiety with the consequent formation of a double bond. While there have been no reports on the further metabolism of the disaccharide, the widespread distribution of this type of hyaluronidase in bacteria suggests that the disaccharide may be further utilized. Figure 6 suggests several possibilities. Hydrolysis, followed by reduction of the aldehyde group would yield an acid which fits into the Doudoroff-Wood pathway. On the other hand, addition of water to the double bond could result in the formation of any of the uronic acids indi-
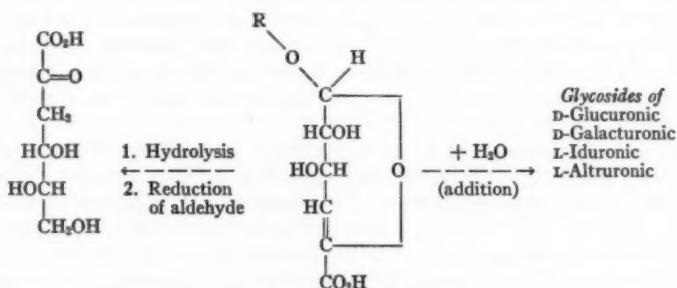
Fig. 6. Possible metabolic routes of unsaturated glucuronide.
(R = N-acetylglucosamine)

cated in Figure 6, depending upon the mechanism of addition. One of the possibilities is L-iduronic acid, which does occur in mammalian tissues.

Hexosamines.—D-glucosamine and its N-acetyl derivatives have been investigated more completely than the related compounds, and the known enzymatic reactions are presented in Figure 7. Studies with C^{14}-glucose demonstrated that the carbon chain of D-glucose was converted without cleavage to that of glucosamine (126 to 130). Lowther & Rogers (131, 132, 133) found that the nitrogen atom of glucosamine was derived from the amide group of L-glutamine. The first cell-free experiments on glucosamine formation were reported by Leloir & Cardini (134), who showed that extracts of *Neurospora crassa* converted hexose-P and glutamine to a hexosamine, presumably glucosamine-6-P. Owing to the presence of isomerase (Reaction 40), no distinction could be made between glucose-6-P and fructose-6-P as the substrate in this reaction. The extract specificity required hexose-P and glutamine, and the hexosamine nitrogen was derived from the amide group of glutamine. Susbsequent work (135) showed that fructose-6-
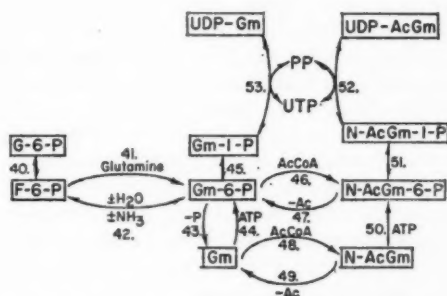
Fig. 7. Metabolism of glucosamine and N-acetyl derivatives.

P rather than glucose-6-P was involved in the reaction using the *N. crassa* enzyme. More recently, the presence of a similar enzyme was reported in rat liver extracts (136), but the authors concluded that glucose-6-P was the substrate rather than fructose-6-P on the basis of relative activity and stability studies; apparently phosphoglucoisomerase was still present in these preparations. In a preliminary communication (137) the enzyme systems from microbial and mammalian cells were compared, and it was concluded that fructose-6-P, not glucose-6-P, is the required substrate in all cases tested. Despite extensive purification of the enzyme from *E. coli* and rat liver, there is no evidence that more than a single step is required for Reaction 41. The enzyme which catalyzes Reaction 41 exhibits no cofactor

requirements, the reaction is apparently irreversible, and the mechanism is still unknown.

Lutwak-Mann (138) studied the dissimilation of glucosamine, D-galactosamine, and their N-acetyl derivatives, by mammalian tissue slices and bacteria; the products were NH_3 and unknown compounds. Apparently the first step in the utilization of glucosamine and N-acetylglucosamine is the phosphorylation of these compounds (Reactions 44 and 50). Except for the specific glucosaminekinase of *Schistosoma mansoni* (139), glucosamine is apparently phosphorylated by the nonspecific glucokinase (140, 141). The product of the reaction was shown to be glucosamine-6-P (142, 143). Glucokinase does not phosphorylate N-acetylglucosamine, but specific kinases are required (140, 144, 145, 146) yielding N-acetylglucosamine-6-P. Kinases for galactosamine and N-acetylgalactosamine are present in mammalian tissue (146); the products here were apparently the 1-phosphate esters.

Early observations (144, 147, 148) indicated that glucosamine-6-P was converted to NH_3 and a hexose-P by extracts from bacteria and rat brain. As a result of the presence of phosphoglucoisomerase, the nature of the hexose-P remained in doubt. Later (149 to 152) it was shown that the products were fructose-6-P and NH_3. While bacterial extracts (150) required no cofactor to catalyze Reaction 42, pig kidney extracts apparently required N-acetylglucosamine-6-P and, in fact, attacked the latter compound very effectively (149). On the basis of these observations, a mechanism for Reaction 42 was proposed for the mammalian enzyme which required participation of N-acetylglucosamine-6-P as an intermediate in the conversion of glucosamine-6-P to fructose-6-P. The seeming discrepancy in mechanisms proposed for Reaction 42 was investigated (153) by comparison of the properties of the purified enzymes obtained from *E. coli* and pig kidney. On the basis of kinetic, specificity, and isotope data, it was concluded that the bacterial and mammalian enzymes acted in the same manner, that N-acetylglucosamine-6-P was not an obligatory intermediate in the reaction, but that N-acetylglucosamine-6-P stimulated both enzymes as did other compounds such as N-acetylgalactosamine-6-P. The purified enzymes exhibited no activity toward galactosamine-6-P, N-acetylgalactosamine-6-P, or N-acetylglucosamine-6-P. The action of crude pig kidney extracts on N-acetylglucosamine-6-P to yield fructose-6-P, NH_3, and acetate was suggested to proceed by Reactions 47 and 42. Reactions 47 is a simple hydrolysis of N-acetylglucosamine-6-P to glucosamine-6-P. While preliminary data indicate the presence of such activity in bacterial extracts, this enzyme has not yet been demonstrated in mammalian tissues. A deacetylase has been described for the conversion of N-acetylglucosamine to glucosamine (Reaction 49) and was found in a number of bacterial but not in mammalian tissue extracts (154). Reaction 42 is distinct from Reaction 41, as indicated by the specificities of the enzymes involved. Further, while Reaction 41 was not demonstrably reversible, Reaction 42 was measurably reversible, although the equilibrium lies well toward fructose-6-P formation.

The acetylation of glucosamine was first noted with pigeon liver extracts and AcCoA (155), where it proceeded to a limited extent. Subsequently, it was concluded (156) that this acetylation was probably catalyzed by the non-specific aromatic amine acetylase long known to be present in pigeon liver. *N. crassa* extracts acetylated both glucosamine and glucosamine-6-P (134), although the presence of phosphatases made it impossible to say which of the compounds was the substrate. Reaction 46 was demonstrated (157) with yeast extracts which were inactive with glucosamine. Reactions 46 and 48 (158) were investigated in microbial and mammalian systems and the enzyme which catalyzes Reaction 46 was purified from *N. crassa* extracts. The glucosamine-6-P acetylase was reported to be highly specific for gluco-samine-6-P, the enzyme was widely distributed in nature, and the physio-logical significance of Reaction 48 was questioned.

It may be of interest to note that Reactions 46 and 42 can be coupled. This represents a pathway of synthesis of glucosamine-6-P and N-acetyl-glucosamine-6-P which involves NH_3 rather than glutamine. Thus, a com-bination of fructose-6-P, NH_3, AcCoA, the purified acetylase, and limiting amounts of either the bacterial or mammalian deaminase rapidly produced N-acetylglucosamine-6-P (153). In fact, on the basis of the relative activi-ties of the enzymes which catalyze Reactions 41, 42, and 46 in crude extracts obtained from various sources, it was suggested that the ammonia pathway is potentially a better source of glucosamine derivatives than is the glutamine pathway.

A number of phosphatases will cleave glucosamine-6-P to glucosamine (Reaction 43), although a phosphatase has been obtained from *N. crassa* (159) which exhibits markedly greater activity toward glucosamine-6-P than any of the other hexose phosphates tested.

The conversion of glucosamine-6-P to glucosamine-1-P (Reaction 45) is catalyzed by crystalline phosphoglucomutase (160), although the amount of enzyme required for this conversion is much greater than that for the com-parable conversion of glucose-6-P to glucose-1-P. A similar mutase which acts on the N-acetyl derivatives, Reaction 51, has been reported (161). Finally, the connection between the phosphorylated glucosamines and the uridine nucleotides through the mediation of pyrophosphorylases and UTP (Reactions 52 and 53) has been described. The available data indicate that the pyrophosphorylases which catalyze Reaction 52 and the cleavage of UDP-glucose are different (81, 162, 163). The isolation of UDP-acetyl-glucosamine from plant sources (164) is of interest since glucosamine is relatively rare in higher plants, although it has recently been reported to be a constituent of plant lipides (165).

The enzymatic synthesis of UDP-glucosamine, Reaction 53, (162) leads to the question of the metabolism of the free amino sugar. UDP-glucosamine has not yet been isolated from nature. While the hexosamines generally exist as their N-acetyl derivatives, and occasionally as the N-glycolyl derivative (in the sialic acids), glucosamine is present in heparin, as the N-sulfate

derivative. A polymer of galactosamine, which is only partially N-acetylated, has recently been reported (166); the remaining amino groups are present in the free form. The problem concerning the metabolism of the unsubstituted glucosamine derivatives lies in the unresolved question of enzyme specificity. Thus, glucosamine is phosphorylated by glucokinase (Reaction 44); glucosamine-6-P is converted to glucosamine-1-P by phosphoglucomutase (Reaction 45), although the enzyme is considerably less effective with these substances than with the corresponding D-glucose esters; finally, the enzyme which catalyzes Reaction 53 has not yet been separated from the pyrophosphorylase which acts on UDP-glucose. The physiological significance of Reactions 45 and 53 is still an open question, although the dissimilation of glucosamine by various cells undoubtedly involves its phosphorylation.

The uridine nucleotides can serve as sources for two other amino sugars, galactosamine, and D-mannosamine. The initial report (167) indicating that UDP-acetylglucosamine was converted by rat liver extracts to free N-acetylgalactosamine, uridine monophosphate, and inorganic phosphate, was not confirmed (168). The product was shown to be N-acetylmannosamine (168). In this "epimerization," the acetylamino group at C-2 is apparently the site of enzyme action rather than the hydroxyl group at C-4. The mechanism for this novel biochemical reaction has not yet been determined. The enzymatic reaction apparently requires UDP-acetylglucosamine. UDP-acetylgalactosamine has been isolated from liver (57), although it was not separated from UDP-acetylglucosamine. The enzymatic synthesis of UDP-acetylgalactosamine has not yet been described.[8] There is relatively little information on the metabolism of galactosamine or N-acetylgalactosamine. The kinases were mentioned above; the enzyme which acts on galactosamine has not yet been separated from D-galactokinase. The isolation of the 1-phosphate esters suggests a close similarity in the metabolic pathways of galactosamine and D-galactose. On the other hand, chemically synthesized galactosamine-6-P was enzymatically N-acetylated by preparations which were previously considered to be specific for glucosamine-6-P (143). It is not yet known whether one or two enzymes are involved in the N-acetylation of glucosamine-6-P and galactosamine-6-P. In this connection, the tentative report (169) indicating the presence of an acid-stable phosphate ester of galactosamine in

[8] Drs. Frank and Gladys Maley have recently communicated the following information which has been submitted for publication. A preparation of UDP-glucose-4-epimerase was carried through step 4 of the procedure described by Maxwell (246). Incubation of this enzyme with UDP-acetylglucosamine or UDP-glucosamine yielded UDP-acetylgalactosamine and UDP-galactosamine respectively. It is not yet known whether the epimerizations of the glucose and glucosamine derivatives are catalyzed by one or more enzymes. The isolation of a specific enzyme which interconverts UDP-glucosamine and UDP-galactosamine would probably answer the questions raised above concerning the metabolic significance of Reactions 45 and 53.

cartilage is of interest. If the 6-phosphate esters of galactosamine are shown to be intermediary metabolites, this would be a departure from the known metabolic pathways of D-galactose where the 6-phosphate ester is apparently not involved.

Little is known about the metabolism of mannosamine other than the report cited above (168)[9]. The further utilization of N-acetylmannosamine is discussed in the section on the metabolism of the sialic acids.

The utilization of glucosamine may proceed by pathways other than indicated in Figure 7; for example, D-glucosaminic acid (2-amino-2-deoxy-D-gluconic acid) or its phosphate ester may be involved. Thus, whole cells or cell-free extracts obtained from *Pseudomonas fluorescens* quantitatively converted glucosamine to D-glucosaminic acid (170), although these cells were apparently unable to utilize the D-glucosaminic acid. D-Glucosaminic acid can be utilized by mammals and certain bacteria (171). The oxidation of glucosamine appears to be catalyzed by an oxidase similar to glucose oxidase but of different substrate specificity, since the latter enzyme does not significantly oxidize glucosamine (172). D-Glucosaminic acid is rapidly utilized by bacteria which have been grown on this compound. Extracts obtained from such bacteria (173) catalyzed the following reaction : D-glucosaminic acid $\rightarrow$ 2-keto-3-deoxy-D-gluconic acid $+ NH_3$. Similar results were reported by Imanaga (174), although the keto acid was not definitely characterized in this case. The keto acid was further metabolized in the presence of ATP, presumably through the Doudoroff-Wood pathway previously discussed.

The sialic acids.—The sialic acids have been comprehensively reviewed by Whelan (175) and will not be discussed here except for subsequent developments in this field. A uniform system of nomenclature was recently proposed (176) ; sialic acid is to be considered a class name and the specific compounds will be named as derivatives of neuraminic acid.

The structure of neuraminic acid is proposed to be I of Figure 8 (177); the previous formulation was II. Neuraminic acid per se has not been isolated. The sialic acids include substances where R equals acetyl or glycolyl. In addition to the N-acyl derivatives, two O-N-diacetyl derivatives are known. As shown in structure III, N-acetylneuraminic acid may be considered an aldol condensation product of pyruvic acid and an N-acetylhexosamine. Chemical studies on the degradation (178, 179) of N-acetylneuraminic acid showed that the compound was cleaved under alkaline conditions to pyruvic acid and N-acetylglucosamine. The synthesis of the compound was also reported by treatment of oxaloacetic acid and N-acetylglucosamine under alkaline conditions (180) and N-acetylneuraminic acid was isolated, al-

[9] Hexokinase and ATP rapidly phosphorylate mannosamine and yield an acid stable product. The product was isolated and exhibited the proper analyses for mannosamine-P, presumably mannosamine-6-P (unpublished studies of Bartholomew, Comb & Roseman).

though in low yield. Heimer & Meyer (181) reported that extracts of *Vibrio cholerae* slowly cleaved N-acetylneuraminic acid to pyruvate and N-acetylglucosamine. The N-acetylhexosamine was characterized by paper chromatography and colorimetric reactions. Subsequently an enzyme was isolated (177) from *Clostridium perfringens* which cleaved N-acetylneuraminic acid in the following manner: N-acetylneuraminic acid ⇌ N-acetylmannosamine + pyruvate. This is the first report of the natural occurrence of a derivative of mannosamine. The action of the enzyme which cleaves N-acetylneuraminic acid appears to be that of a typical aldolase; the reaction is reversible
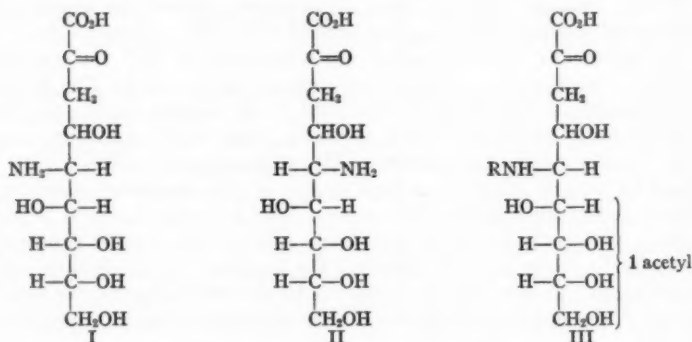
FIG. 8.   I. Neuraminic acid (177)
II. Neuraminic acid (previous formulation)
III. N-acylneuraminic acid

and suggests a mechanism for the biosynthesis of N-acetylneuraminic acid. The enzyme exhibited no activity with N-acetylglucosamine, or N-acetylgalactosamine, but was equally active with synthetic N-acetylmannosamine or with the N-acyl-hexosamine derived from the cleavage of N-acetylneuraminic acid. The cleavage of other sialic acids was also studied and was found to proceed with the N-glycolyl compound at an appreciable rate, but to an insignificant extent with the O,N-diacetyl derivative or with the glycoside, methylneuraminosidic acid.

The hexosamine moiety of N-acetylneuraminic acid was therefore indicated to be N-acetylglucosamine by some workers and N-acetylmannosamine by others. This apparent discrepancy was clarified (182) when it was shown that under the alkaline conditions used in the chemical studies N-acetylglucosamine rapidly equilibrated with N-acetylmannosamine. These results have since been substantiated (183); N-acetylneuraminic acid was converted to a mixture of N-acetylmannosamine and N-acetylglucosamine when the chemical degradation was conducted for a shorter period of time than had been previously used. Further, condensation of oxaloacetate in alkaline solution with

either N-acetylglucosamine or N-acetylmannosamine gave identical yields of authentic N-acetylneuraminic acid (184). The latter report also notes the alkaline epimerization of the N-acetylhexosamines. The alkaline epimerization is the basis of a satisfactory procedure for the preparation of crystalline N-acetylmannosamine (185).

While the evidence outlined above indicates that the hexosamine moiety of the sialic acids is mannosamine, the possibility still exists that the sialic acids are a family of compounds and that sialic acids may be isolated which will contain hexosamines other than mannosamine. The N-acetylneuraminicaldolase reaction suggests the mechanism for formation of N-acetylneuraminic acid, but it is possible that this is a degradative pathway *in vivo* and synthesis occurs in some other manner. A preliminary report (67) indicating the isolation of UDP-nucleotides containing N-acetylneuraminic acid is of interest. Enzymatic "activation" of N-acetylneuraminic acid has not yet been described.

Bacterial cell walls frequently contain muramic acid (186) which is proposed to be a condensation product of lactic acid and N-acetyl-hexosamine. The ether thus obtained bears a resemblance in structure to N-acetylneuraminic acid (187). A preliminary report (188) showing the condensation of phosphoenolpyruvate and UDP-acetylglucosamine in the presence of various bacterial extracts to yield a 3-substituted N-acetylglucosamine derivative is therefore of interest. Reduction of the supposed condensation product would give the muramic acid nucleotide first reported by Park (189). Whether there is any metabolic relationship between the sialic acids and muramic acid is not yet known, although both compounds are present in *E. coli* (177, 186, 190, 191). There is no information concerning the synthesis of the N-glycolyl group and the O-acetyl group of the sialic acids, or the enzymatic synthesis of glycosides containing the sialic acids. The sialic acids can be cleaved from large molecules by hydrolytic enzymes (192, 193). The function of the sialic acids is also unknown. It is of interest, however, that these compounds frequently occur on the periphery of large molecules such as the blood proteins. For example, Popenoe & Drew (192) reported the enzymatic cleavage of sialic acid from orosomucoid leaving the remainder of the protein essentially intact. The isoelectric point of orosomucoid was shifted from pH 2.7 to 5.0—suggesting the important influence of the sialic acids on the physical properties of the polymers in which it occurs.

Activation and transfer of sulfate.—The sulfate problem has been reviewed by Lipmann (194). The conversion of inorganic to organic sulfate involves the following reactions:

$$\text{ATP} + \text{inorganic sulfate(S)} \rightleftharpoons \text{A—R—P—S} + \text{P—P}$$
$$\text{A—R—P—S} + \text{ATP} \rightarrow \text{A—R—P—S} + \text{ADP}$$
$$\underset{\text{(PAPS)}}{\overset{|}{\text{P}}}$$
$$\text{PAPS} + \text{ROH} \rightleftharpoons \text{R—O—S} + \text{PAP}$$

$$\text{ROH} = \text{Sulfate acceptor}$$

While the conversion of sulfate to "active" sulfate (PAPS) has been well characterized and demonstrated in a variety of tissues (195 to 201), the known sulfate acceptors are phenols and steroids. There is, as yet, no report of sulfate transfer to known carbohydrate derivatives. The sulfate acceptor may therefore be the free carbohydrate, a phosphorylated derivative, a nucleotide, or even a polysaccharide. Chlorella produce a sulfated galacto-lipide (202) which is apparently of relatively simple structure, compared with the sulfated polysaccharides, and which may therefore provide useful information in this field.

SYNTHESIS AND TRANSFER OF THE GLYCOSIDIC BOND

General enzymatic mechanisms.—Figure 9 presents the three general reactions by which disaccharides, polysaccharides, etc., are formed, along with related reactions. A compound containing a glycosidic bond (Gl—O—Y) is

$$Gl-O-X + H-O-Y \rightleftharpoons Gl-O-Y + H-O-X$$

X	Y	Enzyme	Reaction No.
Phosphate	H	Phosphatase	
Phosphate	Carbohydrate	Phosphorylase	54
Sugar or Aglycone	H	Glycosidase	55
Sugar or Aglycone	Carbohydrate	Transglycosidase	56
UDP	Carbohydrate;	"Synthetase" or	57
GDP (?)	Aglycones	"Transferase"	

FIG. 9. Glycosyl transfers and related reactions.
(GL = glycosyl residue)

formed by transfer of a carbohydrate moiety from a compound which initially contains a glycosidic bond (Gl—O—X). X can be a phosphate, carbohydrate, or nucleotide diphosphate residue. The enzymatic synthesis of glycose-1-P by the direct action of kinases and ATP or the combined action of kinase and ATP followed by mutases, has already been discussed. The synthesis of the sugar nucleotide diphosphate compounds was also considered. The disaccharides (X = carbohydrate) are derived from either the sugar nucleotides or the sugar phosphates. Thus, the energy for the formation of the Gl—O—X is ultimately derived from ATP, sometimes directly and frequently via several steps.

Reaction 56 is catalyzed by transglycosidases and appear to be more diverse than those catalyzed by the phosphorylases or those involving the nucleotides. The transglycosidases are reviewed elsewhere in this volume and also are discussed in the comprehensive review by Barker & Bourne (203) and the more recent publication of Stacey (204). Transglycosidases are frequently nonspecific (205), so that the *in vitro* synthesis of a glycoside via Reaction 56 may not proceed *in vivo*. Another difficult problem results from the fact that many glycosidases (Reaction 55) exhibit transglycosidase activity under the proper conditions (206). The present line of demarcation

between the two types of enzymes depends upon experimental conditions such as relative concentrations of water and acceptor molecules (H—O—Y), the ratio of transferring to hydrolytic activity, etc. Possibly, a spectrum of enzymes exists rather than the two specific classes, and some of the enzymes presently regarded as glycosidases may be transglycosidases *in vivo*, where conditions favor this activity.

Most of the mucopolysaccharides contain β-glycosidic bonds. On the other hand, the D-glycose-1-phosphates and their corresponding nucleotide diphosphates are generally considered to be of the α-configuration. The mechanism of inversion at C-1 of the glycose residue during the transfer is therefore of interest. There are only a few well-authenticated reports of enzymatic inversions of this sort. Fitting & Doudoroff (69) demonstrated that extracts obtained from *Neisseria meningitidis* converted maltose and inorganic phosphate to glucose and β-glucose-1-P. The reaction was reversible with an equilibrium constant of 4.4 in the direction of maltose synthesis. Similar findings have been noted in the case of cellobiose (207, 208). In this case, α-glucose-1-P and glucose are the products of phosphorolysis. Again, the equilibrium favors disaccharide formation. On the other hand, there is no change in configuration during phosphorolysis of sucrose although a mechanism was suggested (69) involving a double Walden inversion. In the case of sucrose, the equilibrium constant is about 0.05 in the direction of disaccharide formation.

Liver preparations catalyze the transfer of glucuronic acid from UDP-glucuronic acid to a host of acceptors; despite the apparent nonspecificity of this transferase, there have been no reports of transfer to a sugar residue. Isselbacher (209) has reviewed much of the early work in this field. Generally, liver microsomal preparations were used although the enzyme(s) is present in various tissues (210). The enzyme(s) is apparently nonspecific and transfers glucuronic acid to phenolic, alcoholic, carboxylic acid, and amino groups (211). The *p*-aminobenzoylglucuronide isolated from dog urine is suggested (212) to be a furanoside. This product could not be obtained by a simple transfer from UDP-glucuronic acid (which presumably possesses the pyranose structure). The jaundice of the newborn appears to be attributable to an inability to form bilirubin glucuronide. The enzymatic defect in the fetus and newborn is apparently associated with a low level of transferase and UDP-glucose dehydrogenase activities (213 to 217); the activities increased markedly with age in the guinea pig (213). Plant extracts convert UDP-glucose and anthranilic acid to the β-glucoside (218), an analogous reaction to glucuronide formation.

The phosphorolysis of maltose, cellobiose, and sucrose by bacterial extracts was discussed above. However, sucrose phosphorylase was not found in plant extracts. This problem was resolved by experiments which showed that plant extracts catalyzed the transfer of glucose from UDP-glucose to either fructose or fructose-6-P (219, 220). The enzymes were different,

yielding sucrose in one case and sucrose-P in the other; the enzymes have
not been completely separated. The equilibrium constant is about five in
favor of sucrose synthesis, which is in marked contrast to the phosphorylase.
The enzyme is present in pea seed extracts; sucrose formation was noted
only with free fructose (221).

An analogous reaction to the synthesis of sucrose and sucrose-phosphate,
is the enzymatic synthesis of trehalose-P by an enzyme from yeast (222).
The reaction involves the transfer of glucose from UDP-glucose to glucose-
6-P yielding UDP plus trehalose-P; the equilibrium constant at pH 6.6 was
about 40, and calculated to be 192 at pH 7.4. Surprisingly, reversibility of
this reaction could not be detected despite attempts to "pull" the reaction
toward the left. The yeast transferase preparation also contained a phos-
phatase which was specific for trehalose phosphate.

A glycosyl transferase has also been obtained from bovine mammary
tissue (223). Incubation of extracts from this tissue with UDP-glucose and
glucose-1-P gave lactose-1-P, presumably through the intermediate forma-
tion of UDP-galactose. Neither glucose nor glucose-6-P could substitute for
glucose-1-P as acceptors. Lactose is a β-galactoside, which suggests a
Walden inversion during the transfer reaction.

Polysaccharide synthesis.—The first clear example of the participation of
sugar nucleotides in polysaccharide synthesis was the report of Glaser &
Brown (224) on the synthesis of chitin by cell-free extracts of *N. crassa*
utilizing UDP-acetylglucosamine as substrate. Both the particulate and sol-
uble enzyme preparations required chitodextrins as "primers," and the par-
ticulate preparation was greatly stimulated by N-acetylglucosamine. The
product of the reaction was characterized as chitin by enzymatic and acid
hydrolytic techniques which gave either N-acetylglucosamine or its oligo-
saccharides. A net synthesis of polysaccharide was also observed. In view of
the reversibility data cited above with the disaccharides, it is surprising to
note that the "chitin synthetase" system is apparently reversible. While the
synthesis of chitin may involve more than a single step, transglycosidation
is probably not involved. This type of study was extended to cellulose syn-
thesis utilizing extracts of *Acetobacter xylinum*, labeled UDP-glucose, and
cellodextrins as "primers" (225). The best enzyme preparations were pres-
ent in particulate fractions although a labile, soluble preparation was also
obtained. Cellulose was characterized in a similar manner to the procedures
used for chitin. Net synthesis was not reported, and the reaction was not
demonstrably reversible. The mechanism of fibril formation has not yet been
clarified, either in the case of chitin or cellulose formation. The available
evidence (226) suggests that, in the case of cellulose at least, fibril formation
takes place extracellularly "remote from the bacterial cell wall." The trans-
fer of glycosyl residues from the uridine nucleotides to the ends of pre-
existing chains in both cases discussed above involves a Walden inversion
and the formation of a β-1,4-glycosidic bond. In contrast to the results ob-

tained with C^{14}-labeled UDP-glucose (225), cellulose synthesis was not observed with unlabeled UDP-glucose (or other glucose phosphates) either with dried cells, homogenates, or extracts of *A. xylinum* (227, 228). Net synthesis of cellulose in the bacterial systems remains to be demonstrated.

A soluble enzyme from liver was reported (229) which produces glycogen from UDP-glucose and either glycogen or soluble starch as "primer." In this case, net synthesis of glycogen was observed. It has been suggested[10] that the transferase enzyme which yields glycogen may act in a synthetic capacity, while phosphorylase acts in the degradation of glycogen. Possibly the transferase enzyme is involved in the formation of glucose polymers which closely resemble but are not equivalent to glycogen (230, 231). Further, glycogen is apparently heterogeneous (37) and the transferase and phosphorylase enzymes may each be involved in the synthesis of only certain components of glycogen.

The enzymatic synthesis of another β-glucan (1,3-glycosidic bonds) has been reported (232) with extracts of plants and UDP-glucose. The active fractions were particulates which yielded active but unstable soluble fractions. The results indicated a high degree of efficiency of transfer from UDP-glucose to water-insoluble polymer(s). Glucose and a variety of glucose-containing compounds markedly stimulated the preparation, which led the authors to suggest the possibility that more than a single step was involved in synthesis of the polymer. Preparations were obtained from a variety of plants which catalyzed the formation of the β-1,3-linked glucan, but no preparations which produced the corresponding 1,4-isomer, cellulose. The pathway of cellulose synthesis in plants remains to be demonstrated.

The problem of the synthesis of a more complex polysaccharide, hyaluronic acid, is even less well defined than the simpler polysaccharides discussed above. Originally, the biosynthesis of hyaluronic acid was noted using labeled UDP-acetylglucosamine, UDP-glucuronic acid and extracts of Rous sarcoma (233). However, there was relatively little incorporation of label, and significant losses of radioactivity were found on reprecipitation of the hyaluronic acid or on electrodialysis. A preliminary report (234) indicates the enzymatic synthesis of hyaluronic acid utilizing similar substrates but with extracts obtained from Group A streptococci. This system is apparently much more efficient than the Rous sarcoma extracts. This is another example in which β-linked polymers are formed with the α-sugar nucleotides. The detailed mechanism of hyaluronic acid synthesis awaits clarification.

Biosynthesis of sulfated polysaccharides.—Only a few of the recent publications in this field will be discussed. The pioneer work of Dziewiatkowski & Bostrom has been reviewed (235, 236).

Preliminary reports indicate that extracts of embryonic cartilage are capable of forming chondroitin sulfate when incubated with ATP and in-

[10] Dr. L. F. Leloir, private communication.

organic sulfate (237, 238). This enzyme system is soluble after high speed centrifugation and is stable to lyophilization. These surprising results indicate that all of the carbon sources for the formation of polysaccharide are present in the extracts. While characterization of the product as chondroitin sulfate is still preliminary, confirmation and extension of these findings should lead to an understanding of the mechanism of synthesis of one of the more complex connective tissue mucopolysaccharides. At the present time, there is no information on the nature of the intermediates involved in the synthesis.

The only available reports on heparin biosynthesis indicate that labeled sulfate or glucose can be incorporated into heparin; these experiments were conducted either *in vivo*, or with mast cell tumor slices (239, 240, 241).

Synthesis of complex polysaccharides.—The information summarized above indicates the extent of our information on the biosynthesis of certain of the homopolysaccharides and some of the simpler heteropolysaccharides. These studies suggest the difficulty in approaching the problems of biosynthesis of the complex heteropolysaccharides such as the bacterial and plant gum polysaccharides which frequently contain more than three types of monosaccharides, phosphate, sulfate, etc. If these polymers are composed of simple repeating units, then enzymatic synthesis may proceed along the lines outlined above. However, many of these polysaccharides appear to be much more complex, i.e., there is no simple repeating unit. The problem of biosynthesis of the latter substances then becomes as difficult to visualize as that of protein, DNA, or mucolipide syntheses. On these grounds, it seems reasonable to suggest that complex polysaccharide synthesis may require some preformed template such as RNA or DNA, and does not proceed by simple polymerization as in the case of glycogen. Perhaps glycosyl residues are transferred to a polynucleotide chain (either to phosphate or ribose moieties), analogous to the transfer of amino acid residues in protein synthesis.

MISCELLANEOUS PROBLEMS

This review has stressed enzymatic reactions involved in the synthesis of the carbohydrate constituents of connective tissue. Numerous other problems await investigation in this field. For example, while there is a good deal of information on the metabolism of D-glucuronic acid, little is known about the metabolism of many of the other connective tissue monosaccharides. Only a very modest beginning has been made toward an understanding of the mechanism of synthesis of the mucopolysaccharides. The metabolism of glyco- or mucoproteins is essentially unknown; an *in vivo* study (242) suggests an approach to this field. Various hormones, vitamins, etc., are known to produce marked changes in the chemical and physical nature of connective tissue, including morphological changes, but the biochemical explanations for these effects are not yet available.

A number of diseases are classified as "connective tissue diseases"; presumably these are biochemical diseases in the sense that the normal metabolic patterns of connective tissue have changed. There is no information about the enzymatic disorders, and little or none on the chemical composition of the connective tissue which indicates how the tissues in these diseases differ from the normal.

Connective tissue can be cultivated by subcutaneous implantation of plastic sponges or by injection of suitable agents such as carageenin. These techniques are extremely useful for study of the biochemistry of connective tissue; thus, ascorbic acid was shown to act locally in the synthesis of collagen (243). The lipide content of the sponges varies with the species but can represent most of the tissue dry weight (244). Little is known about connective tissue lipides and this should prove a fruitful area for investigation, particularly since many of the monosaccharides discussed above occur in complex lipides such as the cerebrosides, gangliosides, etc.

With few exceptions (e.g., hyaluronic acid presumably acts as a lubricant in the joints by increasing the viscosity of the joint fluid), there is almost no information on the function of connective tissue "mucoid" substances. Some possible roles of the mucopolysaccharides include maintenance of tissue structure, resistance to mechanical stress, transport of metabolites to and from cells, and water binding (245). In view of the wide distribution of these substances in the body, they may have other functions which are not presently obvious, but are much more important. For example, response of the body to many immunological stimuli may be manifested primarily in the connective tissue; a chemical explanation for these phenomena must be sought. This review has not considered the fibrous proteins. While there is considerable information on the metabolism of these substances, the relationship between the proteins and the "mucoid" substances remains to be defined.

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NEUROCHEMISTRY[1,2]

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This review is the first in this publication on the subject of neurochemistry, and its aims will therefore be twofold. First, the general topic of neurochemistry will be outlined and recent pertinent reviews cited to provide an orientation for the remainder of the review. Second, selected topics with which the author is most familiar will be reviewed either to provide a summary on a topic which has not been reviewed recently or to bring up to date the subject of another recent review.

GENERAL OUTLINE OF NEUROCHEMISTRY

The term "neurochemistry" has recently become widely and loosely used because of greatly increased interest in the chemical aspects of nervous and mental diseases. In the strict sense, however, we feel that the term should be used only to describe the biochemistry of the nervous system itself to the exclusion of chemical changes in other parts of the body during mental disease, the effects of nervous activity on the rest of the organism, etc. So limited, the topic of neurochemistry can, for the purposes of this review, be conveniently divided into the following three sections: the chemical structure of nervous tissues; the metabolism of nervous tissues; and the chemical basis of nervous function.

Because of the increased activity in the field, a number of comprehensive references have been published in the past few years which provide a good background for subsequent reviews. These include several books (36, 68, 115) and a new periodical, *The Journal of Neurochemistry.* Two series of symposia have been started and the publications containing their proceedings provide further reference material in the field. In one of these series, the International Neurochemical Symposia, the subject for the first meeting was "Biochemistry of the Developing Nervous System" (180); for the second, it was "Metabolism of the Nervous System" (142); and for the third, "Chemical Pathology of the Nervous System" (47). In the other

[1] The survey of the literature pertaining to this review was completed by January 1, 1959.

[2] The following abbreviations are used: ATP for adenosine triphosphate; CDP for cytidine diphosphate; DNA for deoxyribonucleic acid; NPN for nonprotein nitrogen; PAS for periodic acid Schiff; RNA for ribonucleic acid; UDP for uridine diphosphate.

series, the subjects have been "Neurochemistry" (96), "Ultrastructure and Cellular Chemistry" (181), "The Biological Activity of Psychopharmacological Agents" (138), and "Myelin" (138). The symposium on "Biochemistry of the Central Nervous System" at the Fourth International Congress of Biochemistry has also provided very recent reviews.

With regard to the detailed aspects of the chemical structure of nervous tissues, the material on brain lipides has been reviewed recently (104) and will be brought up to date in the present review. To our knowledge, no recent comprehensive review on the chemistry of brain proteins has appeared and this subject will be reviewed in more detail. Of the other main structural components, the present status of knowledge of carbohydrates and polysaccharides has been summarized by Brante (9, 10), and of nucleic acids in the proceedings of the Aarhus Symposium by various authors (142). All aspects of metabolism were also covered in this symposium, lipide metabolism by Rossiter (151), and protein metabolism by Waelsch (182). The latter subject has been reviewed more recently by Richter (143), and recent developments in both fields will be reviewed here. Reviews on the various aspects of the chemical basis of nervous function and the general problem of the transport of ions across nerve membranes have been contributed by Hebb (66), Nachmansohn (122), Ussing (168), and Keynes (86, 87).

Several other aspects of neurochemistry have been the object of considerable research in recent years, and, consequently, the subject of comprehensive reviews. Woodbury (187) has discussed the work on the interrelationship of adrenal cortical hormones and the central nervous system, and the work in the very active field of investigating the metabolic interrelationships and possible physiological role of γ-aminobutyric acid has been reviewed by Elliott (35). Present status of knowledge concerning the cerebrospinal fluid was summarized by Holmes & Tower (73) and was the subject of a recent symposium (19).

THE CHEMICAL STRUCTURE OF NERVOUS TISSUE

As work progresses toward the elucidation of the chemical structure of the various histologically observed constituents of nervous tissues, emphasis is being placed on use of mild techniques in an attempt to preserve the tissue constituents in their "native state." As this is done, it is becoming obvious that all the materials—lipides, proteins, polysaccharides, and nucleic acids—are associated with one another in a distressingly wide variety of linkages. While a good start has been made toward establishing the structure of many of the simpler types of molecules which make up these complexes, e.g., the elucidation of the chemistry of brain lipides, the surface is just being scratched on the next step, the demonstration of the smaller molecules in their complexes, and the investigation of how the constituents of the complexes are associated.

LIPIDES

More is known of the chemical structure of the lipide constituents of nervous tissues than of other structural constituents. This knowledge was summarized a short time ago (104), and recent work on general lipide chemistry is discussed in another chapter of the present volume. However, we wish to mention a few important facts about nervous tissue lipides which have been established lately.

Plasmalogens.—Recent work has shown that at least the major part of the "native" plasmalogens of brain tissue, as in other tissues, have one aldehyde and one fatty acid substituted on the glycerylphosphoryl moiety and that the aldehyde is attached in an α,β-unsaturated ether linkage. Thus Rapport & Franzel (141) have extended their studies on halogenation to brain tissue plasmalogens and confirmed previous findings that Br_2, I_2, and H_2 added to them in a manner to be expected for the vinyl ether linkage. In addition, Debuch (28) has shown by ozonizing a purified preparation of human brain ethanolamine plasmalogens and then oxidizing and separating the products that primarily C_{15} and C_{17} monocarboxylic acids are formed, as would be expected from this same configuration. Further confirmation of this structure was obtained by Blietz (5), who separated a tritium-labelled aldehyde from the products of the catalytic cleavage of "native" brain plasmalogen by $HgCl_2$ in tritium water. Whether these brain lipides have β-substituted aldehydes, as Gray (60) has found in ox heart, or α-substituted ones, as have been demonstrated in pig heart (111), remains to be seen.

Fatty acids.—The various fatty acids known to be present in brain lipides were tabulated in the previous review (104). Since that time, in continuation of extensive work on the identification of the fatty acids of brain glycerophosphatides, Klenk & Montag (92) have isolated and characterized an additional tetraenoic acid, $\Delta\text{-}^{9,12,15,18}\text{-}n\text{-tetracosanetetraenoic}$ acid. Further studies on the C_{22} polyenoic fraction from ox brain (93) have shown that it consists of 5 per cent dienoic or unidentified acids, 8 per cent trienoic, 38 per cent tetraenoic, 6 per cent pentaenoic, and 43 per cent hexaenoic acids. The trienoic acid is primarily $\Delta\text{-}^{7,10,13}\text{-docosanetrienoic}$ acid and the pentaenoic fraction consists of 70 to 80 per cent $\Delta\text{-}^{4,7,10,13,16}$ and 20 to 30 per cent $\Delta\text{-}^{7,10,13,16,19}$ acids.

Phosphoinositides.—In previous reviews (51, 104), Folch & LeBaron held to the idea that only two forms of combined inositol occurred in brain, diphosphoinositide and phosphatidopeptides, and that the inositol in these substances was combined as inositol diphosphate only. Several workers had reported (62, 77) that inositol monophosphate could be demonstrated in hydrolysates of brain lipides or of extracts containing the protein-bound phosphorus of brain. Nevertheless, it was felt that a careful analysis of the conditions of hydrolysis was necessary before it could be definitely concluded that inositol monophosphate occurred in intact lipides and was not simply a partial hydrolysis product from lipides in which the inositol was, in fact,

doubly esterified with phosphate. Two recent papers now make it necessary to re-evaluate the previous conclusions.

The first of these is a preliminary report by Hörhammer *et al.* (75) that they have succeeded in separating by countercurrent distribution the lipides of a purified "Folch Fraction I" (45) into two inositol-containing fractions in a system of 97 per cent methanol:petroleum ether:benzene, 3:1:3. The first of these fractions was separated by further countercurrent distribution into phophatidyl serine and a substance which exhibited the same behavior as liver monophosphoinositide when chromatographed on paper. The phosphorus and inositol contents of this substance were also correct for a monophosphoinositide. The second inositol-containing fraction had the same phosphorus and inositol content, infrared spectrum, and paper chromatographic behavior as the brain diphosphoinositide previously isolated by Folch (45). A lysomonophosphoinositide was also separated from the first inositol-containing fraction.

The second report indicating the existence of monophosphoinositide in brain is less direct (71). In this instance the hydrolysis products of brain lipides were analyzed by chromatography. When the hydrolysis of lipides labelled with ^{32}P was carried out under conditions in which brain diphosphoinositide yields primarily inositol metadiphosphate (46), no radioactivity was found in the spot shown by carrier inositol diphosphate. Considerable radioactivity did occur in a spot corresponding to inositol monophosphate, however. These results would seem to indicate the presence in the tissue of a lipide metabolically distinct from diphosphoinositide and yielding inositol monophosphate under the same conditions of hydrolysis in which diphosphoinositide yields inositol diphosphate. Taken together with the report of Hörhammer and co-workers, this would seem to suggest the existence in brain of a third type of inositol lipide, possibly similar to the phosphatidyl inositol which has been obtained from other sources (39, 183).

Phosphatidic acids.—Although the presence in mammalian tissues of phosphatidic acids, at least in minute amounts, has long been postulated, these substances have yet to be isolated from this source and completely characterized. Perhaps the best evidence for their existence is given in a recent paper by Hokin & Hokin (70), who included brain tissue in their studies. Taking advantage of the fact that the fraction postulated to be phosphatidic acids had a relatively high rate of incorporation of ^{32}P, they studied the behavior of the metabolically labelled substances when chromatographed with carrier phosphatidic acids. They found that the radioactivity was diluted uniformly when chromatographed with cabbage phosphatidic acids as carrier, but that this was not true when synthetic saturated phosphatidic acids were used as carrier, although the R_F was similar. After hydrogenation, the labelled substances were diluted with the synthetic substances. These facts, combined with the finding that 85 per cent of ^{32}P was obtained as glycerophosphate after hydrolysis, are taken as indicative that at

least minute amounts of unsaturated phosphatidic acids do exist in brain tissue.

Mucolipides.—Another field in which active work is being carried on is the study of the nature of a group of less well defined materials, the mucolipides. This classification is used to include those substances which have been named gangliosides (91), strandin (48), or mucolipides (150), which are characterized by solubility in water and relative instability to acids, and which possibly have a high molecular weight. They are constituted by fatty acids, sphingosine or a similar base, galactose, glucose, galactosamine, and sialic acids.

Since the previous review (104), Svennerholm (160) has reported improvements in the methods for quantitative determination of these substances in nervous tissue and given data for senile human brains. In addition, reports have appeared on chemical studies which had previously been described only briefly. Thus Bogoch (6) has extended the studies on previously reported strandin preparations (56) to include the isolation and characterization of a glucocerebroside from the products of partial hydrolysis. Another fragment suggested to be a hexodicerebroside was also obtained. Using this same preparation, which is homogeneous by many criteria, Meltzer (118) showed that as many as eight fractions might be obtained by the new technique of three phase countercurrent distribution. These fractions apparently had similar chemical compositions but slightly different physical properties and might possibly be different degrees of polymerization of the same monomeric unit, different salts of the same polymeric substance, or both.

Rosenberg & Chargaff (149) have also reported more details and analyses of a mucolipide preparation previously reported only in a preliminary manner (148). These workers obtained a substance which contained substantial amounts of combined amino acids and was homogeneous by ultracentrifugal and electrophoretic analysis. In contrast, Folch and co-workers (48) found that their preparations were homogeneous only when the amino acids had been removed by further purification. It is now apparent from all these studies that the mucolipides are very complex high molecular weight associations of polymers and that minor variations in preparative and purification procedures result in differences in the products. Undoubtedly, there are a number of closely similar types of these substances in the tissue. As a consequence, it will be difficult to prepare identical substances by varying techniques and to compare them.

Structural Chemistry of Brain Proteins

While work on nervous tissue has been in the forefront of lipide chemistry, this tissue is a relatively poor subject for studying proteins and much less has been accomplished with brain proteins than with proteins from other tissues, notably muscle and blood. This results in large part from the relatively high lipide content in most nervous tissues and to the fact that

most of the neural proteins exist as complexes with these lipides and with other tissue constituents. No comprehensive review on the structural aspects of the proteins of the nervous system has appeared in recent years, perhaps because only very recently has much work been undertaken. The results which have been obtained are difficult to correlate because many of the experiments have been done by very diverse methods and, even when the basic method is the same, in many cases the exact conditions vary enough to make comparison with other work difficult.

For the purposes of this review, the work can be divided according to the general solubility properties of the fractions, as demonstrated by the procedure used to obtain them. These classifications are: (a) proteins soluble in aqueous media (these studies include the bulk of the work); (b) proteins complexed with lipides in such a way that they are soluble in nonpolar solvents; (c) residual insoluble proteins; and (d) total trichloroacetic acid precipitable proteins [this includes much of the metabolic work and some studies on the amino acid composition (21) and enzyme resistance (17) of the proteins].

Proteins extracted in aqueous media.—Although one ordinarily considers many of the proteins of a tissue to be among its water-soluble constituents, the relatively large proportion of lipides in nervous tissues renders the mechanics of isolation of proteins and protein complexes in aqueous media more difficult than usual. One procedure for fractionation of water-soluble proteins was reported some time ago by Palladin & Goryukhina (125), who extracted in succession with water, 4.5 per cent KCl, and 0.1 N NaOH, but it is only recently that more carefully controlled studies of the effects of various conditions on the extraction of brain proteins have been reported. The effects of salt concentration and pH in such extractions have recently been investigated (105), and it was found that in both gray and white matter, the maximum extraction of trichloracetic acid-precipitable nitrogen and phosphorus occurred at an ionic strength of about 3.0 and pH of 6 to 9. Preliminary analysis of the material obtained under conditions of maximal extraction showed that upon exhaustive dialysis a relatively small amount of lipide-free albumins stayed in solution, while the bulk of the extracted solids, containing about one-fourth to one-fifth lipide, were precipitated. Approximately one-quarter of the lipides was cholesterol. Similar preparations were obtained from gray or white matter, although the total amounts yielded by gray matter were considerably higher.

Polyakova & Gotovtseva (131) have investigated the optimal conditions for extracting proteins to be used for further study by paper electrophoresis. In this case, maximum yields were not desired, but rather an extract which would give a good electrophoretic pattern. After studying extraction by succinate, acetate, barbital, or borate buffers at pH's from 3.6 to 9.2, it was concluded that extraction with physiological saline followed with clarification by freezing rather than lyophilization gave the best results.

In addition to the work just described, several apparently distinct water-

soluble proteins have been studied. Maxfield & Hartley have isolated and investigated the fibrous protein of the axoplasm of squid and lobster nerves [(114) and previous papers, see also summary by Schmitt (152)]. For the most part, this work has consisted of studies of the physical characteristics of the protein by use of light scattering, ultracentrifugation, electrophoresis, and viscosimetry. The amino acid composition of the lobster nerve preparation has also been reported (94). The results indicate that this fibrous protein, which is assumed to be the axon filaments, is reversibly dissociated into elements of smaller molecular weight by changes in the ionic environment. This dissociation is apparently the result of a decrease in the diameter rather than the length of the fibers.

In a second study, aimed at the elucidation of the role of copper in certain degenerative diseases, Porter & Folch (135) have separated the copper-containing proteins of brain into three fractions by extracting with aqueous salt solutions at various pH and ionic strengths. From one of these fractions, extracted in 0.1 M NaAc buffer at pH 4.5, a purified preparation—85 per cent homogeneous by ultracentrifugation and containing 0.25 to 0.5 per cent copper—was prepared in two different ways (134, 136).

In other studies, Dmitriev (30, 31) has investigated the role of phosphorus- and sulfur-containing amino acids and lysine in proteins extracted by hot water, and Roboz & co-workers (146) have isolated a collagenlike protein from spinal cord by precipitation with salts from the water-soluble undializable material obtained after autoclaving. Cholesterol-containing brain proteins have been investigated by Konnikova (95) and Eperjessy et al. (37), and the various phosphorus-containing brain protein complexes studied by Brik (12), Broun (13), and Bulankin et al. (14). An incidental finding during work designed to prepare phosphatidopeptides (vide infra) by a less severe procedure (106) was that there exists in brain a protein fraction which is converted to water-soluble form after the protein residue is washed and treated with very dilute acid. This fraction is extremely stable to heat, readily digested by trypsin, and it becomes very viscous in the presence of small concentrations of salts.

The most extensive recent work on brain proteins soluble in aqueous media has been the development of studies using electrophoresis. These studies have made use of both paper and free boundary electrophoresis and recently the use of agar as a medium has been reported (172). For study by the free boundary technique, proteins were extracted by NaCl (123), by Michaelis buffer at pH 8.1 (85), and by veronal buffer at pH 8.6 (79, 80). Eight or nine fractions were obtained and, when compared to the mobility of serum albumin, two or three travelled faster, one had the mobility of albumin and the rest were slower. Keup (85) reported the relative amounts of these fractions in different areas of pig brain. Robuschi & Benassi (147 and previous papers) have compared several methods of extraction using both paper and free boundary electrophoresis. They noted changes according to the severity of the methods of extraction and the degree of autolysis.

With regard to the studies using only paper electrophoresis, although Kaps reported in an earlier paper (81) that only two fractions, with mobilities similar to serum β-globulins were obtained in water extracts of brains, subsequent work in a number of laboratories has demonstrated the same eight or nine fractions noted above. This was true for extracts made with 0.25 M sucrose (16), veronal-acetate buffer at pH 8.6 (69), Barbital-citrate buffer at pH 8.6 (144), phosphate buffer at pH 7.2 (164), and water and physiological saline (126, 130). In several of these studies differential staining was used to indicate the presence in some of these bands of possible complex proteins containing lipides, nucleic acids, or polysaccharides. The proportions of the different fractions in various areas of the brain are also reported. However considerable variation in extraction techniques and in the conditions for the electrophoresis make it impractical to attempt correlation of the various reports at the present time. Two recent studies on the conditions of extraction of water-soluble brain proteins may help to standardize future techniques (105, 131).

The proteins of peripheral nerve have also been the object of recent electrophoretic studies. Two independent reports (120, 129) have shown that the proportion of the fraction having the electrophoretic mobility of serum albumin is much greater in extracts from peripheral nerve than in those from central nervous tissue. In addition, peripheral fibers yield a fraction which migrates toward the cathode under these same conditions (132). This finding of a relatively large proportion of protein behaving as serum albumin has led several workers to attempt to determine whether the protein is truly a constituent of the nerve itself or whether it is a contaminant from blood, connective tissue, or some other extracellular source. The albumin was actually isolated from nerves and characterized first by Deuticke et al. (29) and more recently by Chinese workers (107). The latter showed that the albumin isolated was identical with that of serum by solubilities, electrophoretic behavior, immunoprecipitin reaction against rabbit antisera, and chemical composition, but they concluded that it might have its source in the connective tissues surrounding the nerve bundles. On the other hand, Palladin & Polyakova (127) in a summary of their work concluded that this albumin, although it moves homogeneously with serum albumin during electrophoresis, does not derive from blood, lymph, or connective tissue. This conclusion is founded on microdissection studies and subsequent electrophoretic analysis of the proteins from the separated nerves and connective tissue sheaths.

Two other uses of electrophoresis in studying proteins of the nervous system have been described recently. Sheng et al. (153) employed it to examine a number of nucleoprotein fractions isolated by previously reported methods. They concluded that all the preparations were inhomogeneous and that the nucleic acids, lipides, and proteins could be separated into different components. In the other study, Toschi & Marini-Bettolo (165) analyzed

extracts of the electric organs of Torpedo to localize enzyme activity and chemical components in the five bands which were developed.

Proteins extracted in relatively nonpolar solvents.—Since the demonstration by Folch & Lees (52) that proteolipides, i.e., lipide-protein complexes soluble in relatively nonpolar solvents, could be extracted from brain, few other reports of work on these substances have appeared. Chatagnon *et al.* (18) reported the amino acid composition of both bovine and human brain proteolipides, and, more recently, Uzman & Rosen (169, 170) have studied the lipophilic peptides and proteins which are associated with lipides in the proteolipides. One reproducible protein fraction, termed "neurosclerin," was isolated and its amino acid composition determined. The remaining peptides showed no consistent pattern of amino acid composition and no changes correlated with age. In contrast, neurosclerin, while present in newborn mouse brains in small amounts, showed a definite increase at the time of myelination. The most recent development in the chemistry of proteolipides has been reported by Folch, Lees & Webster (55), who have prepared these substances quantitatively in a single fraction in which almost all of the lipide is combined with protein. The new procedure involves the emulsification of a washed total lipide extract and the isolation of proteolipides as the non-emulsifiable fraction after centrifugation.

A second type of lipide-peptide complex, phosphatidopeptides (50), has also been isolated from brain, although in this case the extracting solvent contained a very small concentration of acid. As originally isolated, these substances were extracted from the insoluble protein residue remaining after brain tissue had been extracted exhaustively with chloroform-methanol and water and then digested with trypsin. The phosphatidopeptides were then extracted with 200 : 100 : 1 chloroform : methanol : concentrated HCl v/v, and were constituted by phosphatides, primarily phosphoinositides, and combined amino acids, the latter apparently in relatively short peptides. More recently, (106), a method has been developed by which analogous substances can be prepared from the tissue without resort to the severe procedures previously used. The resulting substances are chemically similar to the phosphatidopeptides previously described except that they contain slightly more amino acids which are combined in peptides that may possibly have a longer average chain length. The phosphorus of the phosphatide component exhibits considerable chemical lability to dilute acid under certain conditions.

Residual insoluble protein.—Discussion of the residual protein from nervous tissue which remains after extraction with lipide solvents and water involves two subjects, the nature of the proteolytic enzyme-resistant protein residue, classically called neurokeratin, and the nature of the phosphorus remaining in the total residue. Recent studies have shown that the two topics are closely interrelated, however.

Neurokeratin itself was first demonstrated histologically as a protein network in the myelin sheath which remained after the specimen was treated

with lipide solvents and digestive enzymes (38). Preparations of neuro-keratin have also been made from bulk tissue using similar preparative steps, and the resulting substances have been analyzed [see (103) for review.]. However, exact correlation between the total bulk preparation and the histologically demonstrable material is yet to be accomplished. The nature of possible protein precursors of neurokeratin was considered by LeBaron & Folch (103), who attempted to prepare a similar substance by less severe procedures than were used previously. They demonstrated that several of the earlier procedures would result in chemical hydrolysis of some constituents, notably the phosphatidopeptides. Since these earlier procedures also involved lipide extraction by procedures which would split proteolipides, leaving the proteolipide protein with the residue, and since this proteolipide protein is resistant to proteolytic enzymes (52), it appears that bulk preparations of neurokeratin are a mixture of degradation products of proteolipides and other lipide-protein complexes. Whether this is true of the histological entity is yet to be determined. Another recent study on bulk neurokeratin has been reported by Stary & Arat (157). These workers concluded from their studies that the relatively high proportion of sulfur-containing amino acids in neurokeratin, although bound in disulfide crosslinkages to a great extent, were not responsible for the insolubility or resistance to proteases, which neurokeratin exhibits.

Some clues as to the nature of the phosphorus in the insoluble protein residue have come from these investigations on neurokeratin. The classical assumption was that the residual phosphorus, after extraction of lipides and water-soluble materials, was a constituent of either nucleic acids or phosphoproteins. In brain tissue, however, concomitant analyses for ribose, phosphorus, and ultraviolet absorption of purine and pyrimidine bases gave conflicting results when only neutral solvents were used to extract lipides (49, 108). This has been somewhat clarified by the demonstration of the existence of phosphatidopeptides in these residues (50), and a modified procedure for estimating nucleic acids in nervous tissue was developed (108). Nevertheless, there are indications that these substances may not account for all of the unknown phosphorus (77) and considerably more work needs to be done. The existence of phosphoprotein of the classical type has apparently been demonstrated, however (64, 173).

POLYSACCHARIDES

Glycogen.—While the structure of glycogen from other tissues has been studied rather extensively, there are as yet very few such studies on brain glycogen. Goncharova (59) measured the absorption spectrum of the glycogen-iodine complex from rabbit brain and liver and also compared the effect of β-amylase on glycogen isolated from these two sources. Since the extinction coefficient of the iodine complex from brain was lower and the absorption peak was flattened and shifted toward lower wavelengths, and since

β-amylase released less maltose from the brain glycogen, it was concluded that this source had glycogen with more branching and shorter side chains than liver did. This was confirmed by periodate oxidation studies. Khaïkina (88) and Khaïkina & Krachko (89) developed a method for differential extraction of brain glycogen and studied incorporation of ¹⁴C-glucose into the different fractions. About 15 per cent of brain glycogen was found in free form and 60 per cent bound to proteins. The rest was bound to lipides. Fifteen per cent of the protein-bound glycogen could be extracted with dilute NaCl. Free glycogen was exchanged most rapidly and the protein-bound form most slowly. Shimizu & Hamuro (154) have also found evidence for bound and labile forms of brain glycogen, in this case by histochemical staining techniques.

Mucopolysaccharides and mucoids.—The very meager and preliminary knowledge of these substances as they exist in the nervous system has been summarized by Brante (9, 10). Substances representing most of the known types undoubtedly do occur in nervous tissues and the presence of the following constitutents has been demonstrated: hexuronic acids, sulfate, glucosamine, galactosamine, galactose, mannose, fucose, sialic acid, and perhaps traces of glucose. The only report of isolation of substances of this type has been that of hyaluronic acid in peripheral nerve (1).

The potential physical and chemical properties of these substances make it possible that their physiological role in the tissue is quite important, and it is to be hoped that the investigations into their biochemistry will be greatly expanded.

CORRELATION OF CHEMICAL AND MORPHOLOGICAL CONSTITUENTS OF STRUCTURE

State of structural components in situ.—Now that we have reviewed the recent work on the chemistry of the structural constituents of nervous tissue, it is perhaps pertinent to discuss the general problems involved in determining the status of these substances as they exist *in situ*. In most cases, the gap between morphological descriptions and chemical analyses of the nerve tissue structures is still very wide, but in some instances recent work is starting to narrow this gap.

It is obvious from the preceding sections of this review that many of the lipides, proteins, and probably polysaccharides and nucleic acids also, exist in nervous tissue as complexes whose bonds with one another have varying degrees of strength. Very little is yet known about the types of bonds involved, and it is to be hoped that investigations in this field will be forthcoming. One approach is through the investigation of the ionic species of the various constituents and the relative affinities of these various species for other ions. Some recent investigations of Folch, Lees & Sloane Stanley (54) are pertinent in this connection. The three known acidic lipides from brain, phosphatidyl serine, diphosphoinositide, and cerebron sulfuric acid, appar-

ently occur as neutral salts of Na^+, K^+, Ca^{++}, and /or Mg^{++}. However, the relative affinities of the three lipides for the different ions had not been determined, and, consequently, doubt exists as to the validity of concluding that a given lipide exists *in situ* as a given salt, just because it has been isolated as such. Folch, Lees & Sloane Stanley (54) also studied the over-all effect of equilibrating total lipide extracts containing these lipides with aqueous NaCl, KCl, $MgCl_2$, or $CaCl_2$ solutions of various concentrations. It was shown that the presence of salt in the aqueous phase was necessary to prevent some lipides from migrating into it. When sufficient salt was present, the nature of the cation in the lipide phase was determined after successive equilibrations with the different salts. It was found that any one of the four cations could reversibly displace any other from combination with lipides. This was an over-all effect, however, and small amounts of lipides with absolute specificity for Ca^{++} or Mg^{++} could not have been detected. In another type of experiment, Marinetti, Erbland & Stotz (112) succeeded in separating the free acidic form of brain phosphatidyl serine from the Na form by column chromatography. Whether or not these two species existed as such *in situ* is yet to be determined.

Chemical analysis of histological structures.—A second approach to chemical localization is the analysis of isolated histological entities. This field has been quite active in recent years and has been completely reviewed (133, 181). The studies of Lowry & Robins and their co-workers are continuing with an ever increasing degree of resolution, and the reader is referred to previous reviews for details (133, 181). In addition, Albert (3) has reported determination of amino acids and purine and pyrimidine bases in isolated nerve cell nuclei from bovine cerebral cortex. Thirteen amino acids and all the bases usually found as constituents of RNA and DNA were detected. The preponderance of thymine as compared to uracil suggested a higher content of DNA than RNA.

Myelin is the morphological structure of nerve tissue which has been most extensively studied and perhaps represents the one for which the gap between chemical analysis and morphological description is the narrowest. Very extensive morphological studies using electron microscopy, x-ray diffraction, and other physical methods have been conducted [see review by Finean & Robertson (44)] and have led to a proposed chemical structure for peripheral nerve myelin. This structure would consist of thin spiral protein layers separated by a bimolecular layer of lipide, the nonpolar fatty acid chains of the lipide being radially oriented. The more polar portions of the lipides would be associated with the protein layers, one lipide layer associated on each side of each protein layer. From morphological considerations, the alternate protein layers must differ from each other. In a careful analysis of parallel experiments using x-ray diffraction and electron microscopy, the two types of data have been shown to correlate (40). Using the technique of x-ray diffraction, Finean (41) and Finean & Millington (43)

have also studied the effect of hyper- and hypotonic solutions on frog sciatic nerve myelin and compared this with changes brought about by drying. The conclusions drawn were that, while the myelin contained considerable amounts of water, most of this was "bound" in some sort of association with the hydrophilic groups of the lipide and the protein, or both, of the tissue. These phenomena were also studied by electron microscopy (145), and it was shown that the swelling achieved by soaking in hypotonic solution was attributable to separation at only one of the two types of protein layer. This fact, taken with the different reactions of the two types of protein layers to fixatives, is believed to be an indication of chemical differences between the two layer types.

As yet, no conclusive correlations have been obtained between the lipide-protein associations which have been proposed on the basis of physical measurements and morphological description, and the several lipoprotein and proteolipide preparations which have been isolated from nerve tissue. The most obvious correlation is that proteolipides, since their protein moiety is resistant to proteolytic enzymes, are one association of the proposed structure and that the protein moiety is left as the histologically demonstrable neurokeratin. A major block to this conclusion is the independent finding in two different laboratories (42, 53) that proteolipides, as presently prepared, are obtained only from the central nervous system and not from peripheral nerves. At least one preliminary report (186) indicates that much needed histochemical studies are being undertaken to clarify this point.

One of the most extensively investigated aspects of myelin chemistry has been the concomitant study of lipide composition and myelination in young, growing animals. Earlier work on this topic was summarized at the First International Neurochemical Symposium (180). Since that time, further studies with human brain material have been reported by Tingey (163) and by Cumings et al. (24), who obtained expected correlations of lipide composition and degree of myelination. Edgar (34) has studied changes in glyco- and phosphosphingosides in different parts of developing rabbit brains and has found different patterns of change for the two groups of lipides in the various areas. De Almeida et al. (27) have correlated these findings with histochemical studies and obtained evidence suggesting the possibility that sphingolipides were stored intracellularly prior to incorporation into myelin. A similar suggestion that myelin was produced from preformed intracellular lipide has been made by Uzman & Rumley (171) on the basis of an extensive study on developing mice, in which chemical analyses and histological examination were done in parallel. The evidence from these chemical studies was also consistent with their previous hypothesis that water-soluble glyco-lipides were precursors of cerebrosides.

The whole question of the nature, or even the existence, of an intercellular "ground substance" in brain tissue is still very much in doubt. As summarized by Brante (10) and Brierly (11), the consensus seems to be that a

PAS-positive substance does exist either between cells or as part of their exterior processes, but that the actual extent of the space available for such substance may be minimal. In a recent paper, Hess (67) has obtained evidence that the staining substance is not lipide or hyaluronic acid. Also Crevier (23), while not attempting too fine a localization, has studied the degree of staining attributable to both PAS and cholinesterase in developing rats. The latter stain parallels the former at a slightly later time, and this is taken as an indication of possible enzymatic activity of the mucoprotein stained. A preparation of mucoprotein was also made from the tissue and was found to have a high hexosamine content but to be unsulfonated.

METABOLISM OF STRUCTURAL COMPONENTS

On the basis of early work, it was thought that the lipides and proteins of brain were rather inert metabolically. This conclusion was drawn without regard to the very important barriers to exchange of various metabolic intermediaties between nervous tissue and the blood stream. Since these have been taken into consideration and proper experiments undertaken to circumvent the effects of these barriers, it has been found that the lipides and proteins of nervous tissue do have an active metabolism, although it is relatively isolated from the rest of the body. These experiments have included studies of isolated nervous tissue *in vitro*; measurements of incorporation of isotopically labelled substances *in vivo*, using substrates which readily enter the nervous system or injecting them within the nervous system; and demonstrations of the presence of various enzymes cytochemically. The last phase of the work has been reviewed elsewhere (133).

LIPIDE METABOLISM

Since Rossiter's review (151), the work on the metabolism of brain lipides has been extended and the conditions for incorporation of various labelled substrates into the different lipides studied. Thus Majno & Karnovsky (110) measured incorporation *in vitro* of acetate, phosphate, choline, glycerol, or glucose into the total lipide fraction of gray matter, white matter, or peripheral nerve. When peripheral nerves were stimulated, the incorporation from acetate or glucose was increased, but that from phosphate or choline was unchanged (109). Pritchard (137), Miani & Bucciante (119), Marinetti, Witter & Stotz (113), and Hokin & Hokin (72) have reported further experiments on the relative incorporation of phosphate, acetate, glycerol, glycine, serine, ethanolamine, and choline into various phosphatides, their precursors, and their breakdown products. One new facet of this work has been the use of isotopically labelled inositol for studies of incorporation into brain lipides (2, 71). This substrate has been found to be incorporated both *in vivo* and *in vitro*, and Hokin & Hokin (71) showed that it is apparently incorporated in a manner parellel to the incorporation of ^{32}P. Their results indicate that the most active turnover occurs in lipides which may be

monophosphoinositides rather than diphosphoinositides (*vide supra*). Preliminary studies have also indicated that the phosphoinositides combined in phosphatidopeptides are also metabolically active (51, 106, 174).

Further work has been reported on the metabolism of cholesterol in nervous tissue. The incorporation of labelled acetate or pyruvate has been observed *in vivo* (117, 124) and *in vitro* (61), and in the latter study, butyrate, glucose, or mevalonate were also included. Davison and co-workers (25, 26) have studied the fate of injected labelled cholesterol. The results of all these experiments are in agreement and amplify the previous conclusion that cholesterol is synthesized in the central nervous system of young animals, but that synthesis occurs to a much smaller extent, if at all, in adults. The cholesterol once formed is very stable and little turnover occurs over long periods of time.

Possibly the most active field of metabolic investigation recently has been the study of sphingolipide metabolism. The problem has been attacked from a number of different points of view. Brady and co-workers (7, 8) have studied *in vitro* the synthesis of sphingosine by brain tissue and found that serine and palmitaldehyde or palmityl CoA are combined to form sphingosine, probably via dihydrosphingosine. Zabin (188) has shown that these same substances are also used to form ceramides. In the next step toward an intact lipide, Sribney & Kennedy (156) have shown that brain tissue contains an enzyme system which transfers the phosphorylcholine moiety of CDP-choline to the free primary hydroxyl group of a ceramide. They have also studied the isomeric specificity of the system. The incorporation of the carbohydrate moiety of sphingolipides has been studied both *in vivo* in young animals and *in vitro* by Burton and co-workers (15), Moser & Karnovsky (82, 121), Radin *et al.* (140), and Cleland & Kennedy (20). Burton's group (15) has found that either glucose or galactose is incorporated into the lipides, and they identified the *in vitro* product as probably N-cerebronyl-O'-galactosyl sphingosine. With either substrate, the labelling of the hexose moiety was much higher than that of the ceramide, indicating that the hexose was incorporated without prior breakdown. This point was demonstrated more clearly by Moser & Karnovsky (82, 121), who investigated the hexose moieties after hydrolysis and degradation. Ninety-five per cent of the ^{14}C from glucose-6-^{14}C was found in the C-6 of the cerebroside galactose. These workers also investigated the incorporation into mucolipide hexose and hexosamine and found that the majority of the incorporation was without prior degradation. Glucose was incorporated into mucolipide galactose a little more slowly than into cerebroside galactose and into mucolipide glucose or galactosamine at about one-third this rate. Galactose was a better precursor for mucolipides than glucose, but glucose was better than galactose for all other components studied. Cleland & Kennedy (20) showed that labelled UDP-galactose also serves as a precursor for labelled lipide *in vitro*. Radin *et al.* (140) measured the rate of incorporation and turnover of galac-

tose in cerebrosides, mucolipides, and sulfatides and of $^{35}SO_4$ into sulfatides. They demonstrated that, in contrast to the other fractions, the rate of turnover of sulfatides, once synthesized, was neglibile. Incorporation of $^{35}SO_4$ into sulfatides had previously been demonstrated by Holmgard (74) who isolated the labelled lipide.

PROTEIN METABOLISM

The general subject of protein metabolism was well reviewed by Waelsch in 1956 (182), and since that time further studies of protein turnover and of the incorporation of various labelled substrates into the proteins of the nervous system have appeared. The extensive studies of Lajtha and co-workers have been more fully reported (57, 98, 99, 100) and extended to studies with labelled glutamic acid (99). Vladimirov & Urinson (175) have reported the incorporation of labelled glycine into brain proteins. The extensive studies on incorporation of ^{35}S-methionine have been extended to ganglion cells (33) and peripheral nerve (128, 155). In the latter case, the proteins of nerve have been fractionated into salt-soluble, alkali-soluble and residual fractions and the incorporation compared with similar fractions in brain (97). The ratios of incorporation of labelled methionine into the three fractions were about the same for gray matter, white matter, and peripheral nerve, the salt-soluble fraction having the highest incorporation and the residual protein the lowest. However, total incorporation in all fractions was higher in gray matter than in white matter and peripheral nerves had only one-sixth the incorporation of gray matter.

A more specialized aspect of protein metabolism has been the demonstration of an apparent high metabolic rate of the phosphorus of the phosphoprotein fraction. Although earlier work had demonstrated a relatively high turnover rate for the inorganic phosphorus released under the conditions of alkaline hydrolysis usually used to determine phosphoprotein, a great deal of doubt existed about the actual source of this phosphorus since it is obvious that a number of residual phosphorus-containing fractions are present in most preparations (*vide supra*). The actual demonstration of labelled phosphoryl serine (64, 173) in hydrolysates of such protein fractions is a significant step in elucidating the actual structure of at least one active fraction. In his work, Heald took advantage of a previous finding (63) that stimulation of respiring guinea pig cortical slices by electrical pulses increased the incorporation of ^{32}P into the phosphoprotein. When the phosphoprotein from slices treated in this way was separated and hydrolyzed and the hydrolysate was chromatographed with carrier phosphoryl serine, only two radioactive spots were found, those corresponding to phosphoryl serine and to inorganic phosphate. The specific activity of the phosphoryl serine spot was increased in hydrolysates from slices which had been sub-

jected to electrical pulses. Heald has subsequently reported (65) on the intracellular localization of this phosphoprotein.

POLYSACCHARIDE METABOLISM

Glycogen.—Glycogen is not a structural component of nervous tissue in the same sense that lipides and proteins are, but a discussion of its metabolism is included here for the sake of completeness, since its chemistry was discussed above. It has been known for some time that glycogen occurs in brain, and some of the enzymes necessary for its metabolism have been demonstrated in brain. However, the actual extent of metabolic studies on brain tissue which included glycogen have been less extensive than for other tissues. Earlier work has been discussed in McIlwain's monograph (115). With the demonstration that surviving cerebral tissue will resynthesize glycogen (102), a further avenue of investigation was opened and subsequent studies with this *in vitro* system have been reported. In the original study, the level of glycogen in the tissue slices dropped very low before resynthesis could be started, and the rate of resynthesis was rather slow. McIlwain & Tresize (116) have attempted to improve the initial level of glycogen and to accelerate the resynthesis. They found that *in vivo* administration of anesthetics did raise the initial level of glycogen in the slices, but as soon as incubation was started, the level dropped again. The rate of resynthesis was not accelerated by excess glucose or addition of lactate and was somewhat decreased by addition of glutamate, citrate, or glucose-1-phosphate. The only improvements which could be accomplished were in the speed with which the tissues were handled before and after incubation. In confirmation of earlier experiments (102), short-term electrical stimulation had no effect on glycogen level, in this case even in the presence of insulin. Kleinzeller & Rybová (90) have also reported studies using this system and found that the glycogen resynthesis was depressed by a K^+ concentration higher than 40 mM. They also found that it was depressed by glutamate and showed that the depression by 0.01 M glutamate was abolished by 2×10^{-3} M ATP. Aspartate was also found to depress glycogen resynthesis.

The incorporation of ^{14}C-labelled substrates into brain glycogen has been studied recently. Prokhorova (139) reported the incorporation of ^{14}C-glucose and calculated that 5 to 10 per cent of glucose utilization was accounted for by glycogen production. Brain glycogen was estimated to be renewed in 2 to 4 hr., a faster rate than was found for liver. Coxon & Henderson (22) found that brain glycogen was heavily labelled *in vivo* from both ^{14}C-glucose and ^{14}C-bicarbonate. They compared the incorporation from these substrates into brain and muscle glycogen. Another method used for *in vivo* studies with brain glycogen is that developed by Kerr (83), using rapid freezing to study variations caused by treatment of the animal before death. The most recent report of studies of this kind was contributed

by Svorad (161), who measured significantly higher levels of glycogen in the diencephalon, mesencephalon, and medulla oblongata of rats during hypnosis. No changes were noted in the cerebral cortex or cerebellum, and the changes in the other areas were reversed on arousal.

AMMONIA METABOLISM

The subject of protein metabolism in the nervous system has become interrelated with that of ammonia metabolism in these tissues through the suggestion that protein-bound amide groups may be a source of ammonia (176). This possibility has been considered in several recent investigations of the source of the ammonia formed in nervous tissues and is closely related to the over-all metabolism of glutamine and glutamic acid which has been reviewed by Strecker (159). Vrba and co-workers (179), in a follow-up of previous *in vivo* investigations (177), found that protein-bound amide-N of surviving guinea pig cortex slices did decrease significantly on incubation for 4 to 6 hr., but that this decrease could account for only about one-quarter of the ammonia formed. They also found, in contrast to earlier findings of Weil-Malherbe & Green (185) that the increase in NPN did not indicate enough proteolysis to account for much of the ammonia production. On the other hand, Weil-Malherbe & Drysdale (184) were unable to demonstrate an unequivocal decrease in protein-bound amide-N in similar experiments. They also found that when ammonia formation was inhibited by dinitrophenol or anaerobiosis the protein-bound amide-N also decreased, suggesting that it was metabolized in a manner other than conversion to ammonia. These workers also determined total hexosamines in the slices and found no change in their concentration when ammonia was being produced. They thus concluded that hexosamines were not a metabolic source of ammonia. In a third study, Takagaki and co-workers (162) concluded from measurements of ammonia production and glutamic acid loss in guinea pig slices that there were two components of ammonia production, one autolytic and the other resulting from glutamic acid oxidation. The conclusion from all these studies appears to be that the ammonia production of brain tissue is a result of a number of processes, which are probably not uniformly affected by changing metabolic conditions.

INVOLVEMENT OF "STRUCTURAL COMPONENTS" IN FUNCTIONAL ACTIVITY

The discussion of ammonia metabolism brings up the whole topic of the endogenous metabolism of nervous tissue respiring without substrate and its relation to the *in vivo* use of substrates other than glucose either in normal oxidative metabolism or in stimulated and specialized metabolism during function. The basic fact is that, in *in vitro* systems, brain tissue will continue to respire and use some endogenous substrates even after exogenous glucose is exhausted. Vrba & Folbergr (178) have studied this

metabolism and found that free ammonia, NPN, and lipide nitrogen increased during 5 hr. of activity of guinea pig cerebral cortex slices respiring without substrate. On the other hand, nucleoprotein nitrogen, protein nitrogen, and humin nitrogen decreased. The bound pentoses of nucleic acids and the free pentoses also both decreased. This would seem to indicate that nucleic acids and proteins were being used as substrates for oxidative metabolism. Stekiel & Larrabee (158), working with isolated rat ganglia, also found increased ammonia production in the absence of glucose, accompanied by a loss in response to stimulation. The output of ammonia could be reduced again by addition of glucose, but the physiological response was not restored. This evidence, combined with the work reviewed by Geiger (58), demonstrates that under extreme unphysiological conditions, nerve tissue structural constituents are metabolized.

However, the classical view is that glucose is the only substrate utilized by the intact brain as its source of energy under normal conditions (84). Recently a number of new techniques have been employed in studies, the results of which are difficult to explain on this basis. As mentioned above, Vrba has implicated brain proteins in ammonia formation, and he has postulated (177) a cyclic process during which amide-bound nitrogen of proteins is released on activity and combined with glutamic acid to form glutamine. The protein amide bonds are then resynthesized during rest while glutamine decreases. This hypothesis is based on measurements of the various nitrogen compounds in the brains of rats before, during, and after strenuous exercise. Apparent changes in brain proteins during stimulation have also been reported by Ungar and co-workers (166, 167), who measured changes in sulfhydryl groups amperometrically and ionization of side groups by changes in the ultraviolet spectra of extracts of brains and nerves which had been stimulated 20 min. These changes were reversed to normal in 10 to 20 min. of rest. There is also evidence from cytochemical experiments for increased activity of cellular RNA, proteins, and lipides. This has been summarized along with other evidence by Hydén (78). There is certainly ample evidence for the active turnover of various lipide, protein and nucleic acid structural constituents, and the possibility exists that there is some alteration of this turnover on stimulation.

Since the metabolism of the nervous system is largely isolated from that of the rest of the organism, a relatively independent metabolic equilibrium can be postulated for the reactions involving the structural constituents. The main question brought up by the results discussed above is how this equilibrium is related to the oxidative metabolism of the same tissue. At least two possibilities would seem to exist. The first is that the metabolic equilibrium involving the structural components is to a large degree independent of oxidative metabolism and that only minimal interchange of intermediates occurs, primarily from the oxidative reactions toward the

structural equilibrium. The turnover rates in the reactions of this equilibrium are possibly increased by stimulation. The second possibility is that the equilibrium involving structural components is intimately connected with oxidative metabolism by extensive exchange of intermediates and that these interconnected equilibrium reactions are shifted toward oxidative usage of structural components during stimulation. One of the chief exponents of the latter viewpoint has summarized his evidence in a recent review (58). In a perfused cat brain preparation, functional activity, as measured by the excitability of the cortex, can be maintained with glucose-free perfusion fluid under the proper conditions. During this time, decreases in the structural components of the brain can be measured. Similar changes are found when a perfusion fluid containing glucose is used and the preparation is stimulated by electrical pulses. In this case the changes were reversed upon cessation of the stimulus. In addition, it has been shown by Allweis & Magnes (4) that only about one-fourth of administered ^{14}C-glucose in these preparations is oxidized to ^{14}CO$_2$, the remainder being converted to ^{14}C-lactate. This production of ^{14}CO$_2$ accounts for only one-fifth of the O$_2$ consumption of the brain and the rest must be supplied by endogenous substrates.

Larrabee and co-workers, working with isolated rat superior cervical ganglia, have obtained data leading to similar problems. Measurements of O$_2$ uptake (101), glucose uptake, and lactate output (76) were made at 23° (32) and at 36°, at various pH, both with and without stimulation. Upon stimulation at 36°, the increase in glucose utilization was matched by an equivalent increase in lactate production, so the uptake of O$_2$ was assumed to increase at the expense of an endogenous substrate. In contrast, at 23°, the increases upon stimulation varied with pH and the stimulation of lactate production was less than the stimulation of glucose uptake. The changes in rates of O$_2$ consumption, glucose uptake, and lactate production upon stimulation did not occur simultaneously, and when temporal distribution was disregarded, the increase in O$_2$ uptake could be accounted for by glucose uptake after allowance for the extra lactate production.

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BIOCHEMISTRY IN THE U.S.S.R.[1,2]

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Through the co-operative efforts of several agencies of the United States
government, about 60,000 pages per year of key Soviet scientific journals
are now available in English translation. Furthermore, practically all Rus-
sian articles of biochemical and biological interest now include English sum-
maries, although these are of variable merit. A complete list of translated
journals, names of publishers, prices, etc., is available from the National
Science Foundation in Washington. It thus appears that, perhaps, a review
such as this is unnecessary. Opinions were expressed from several quarters
that the translated journals do not appear fast enough, that the editions are
too expensive, or that verbatim translations include articles of low scien-
tific merit along with excellent material, etc. In the opinion of this reviewer,
the increased availability of Russian publications in English translation is
welcome to those who are genuinely interested in the work in which the
U.S.S.R. scientists are engaged.

In this review, as in previous reviews, the author has read all the articles
available to him in the original language. From these a selection has been
made of those which appeared to be the most outstanding or entirely un-
familiar to non-Russian reading biochemists. The selection, unfair as it is to
unmentioned U.S.S.R. biochemists, was also necessitated by the exigencies
of space. In this review, as in previous reviews, the mention of the work of
biochemists not residing in the U.S.S.R. proper carries no political implica-
tions. It appeared appropriate to discuss such work because of its content,
because it was published in Russian or in a language related to it, or because
it was reported in a Russian journal. This explanation is made here in re-
sponse to inquiries received by the author.

Adenosine-triphosphatase.—Engelhardt & Burnasheva (1) fractionated
washed bovine sperm cells by homogenization and differential centrifuga-
tion into fractions consisting of their tails and heads. The tails comprised
one-third of the sperm cells on a weight basis, and the heads two-thirds. In

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[1] The survey of the literature pertaining to this review was concluded in October
1958.

[2] The following abbreviations are used: ACTH for adrenocorticotropin; ATP
for adenosine triphosphate; ATPase for adenosine triphosphatase; DNA for
deoxyribonucleic acid; EDTA for ethylenediaminetetraacetate; Q_{HK} for quotient of
activity of hexokinase; RNA for ribonucleic acid; TCA for trichloroacetic acid.

the tails 25 to 30 per cent of the nitrogen of the whole homogenate was present. The ATPase activity of the sperm cells was localized in the tail fraction, accounting for about 80 per cent of the ATPase activity of the whole homogenate. A protein, called spermosin, which possessed the ATPase activity was isolated from the tail fraction. Seventeen to 22 per cent of the tail homogenate nitrogen was found in spermosin. Spermosin is localized in the tail of the sperm cells, i.e., directly in the contractile apparatus of the motile sperm cell. A comparison of the enzymatic activity (ATPase) of spermosin, isolated either from the tail or from whole bovine sperm cells, did not reveal any difference in the properties of the enzyme preparations.

Ecto-adenosine triphosphatase (ATPase located on the surface of the wall of nucleated cells), previously found in pigeon erythrocytes, is common to all nucleated erythrocytes thus far investigated (2). Ethylenediaminetetraacetate (EDTA) inhibited the activity of ecto-ATPase. In a direct experiment employing EDTA, it was shown that the spontaneous decomposition of ATP within erythrocytes, following their hemolysis, results from the action of ecto-ATPase on ATP. It was further shown that the decomposition of ATP within the erythrocytes, after inhibition of respiration, is not caused by the activity of ecto-ATPase. Employing EDTA, it was possible to show that the entire ATP content of the erythrocyte cell is located in the cytoplasm and that the ATPase activity is bound to structural elements of the cell (2).

Vorobiev (3) has shown that the threads obtained from a complex of myosin with another polyelectrolyte of biological origin, desoxyribose nucleic acid, possess mechanical-chemical properties which resemble those of actomyosin threads (in the presence of 10^{-2} M $CaCl_2$ and 10^{-2} M $MgCl_2$ in 0.05 M KCl, the threads contract under the influence of ATP). Hence, the combination of myosin with actin is not a prerequisite for the mechanical-chemical activity of myosin. In the absence of Ca^{++} the addition of ATP causes lengthening of the threads instead of contraction. This is attributed to a change in the electrochemical properties of the contractile system under the given conditions. In some cases, when different pH ranges, EDTA. etc., were employed, a correlation was established between the deformation value of the threads and the enzymatic activity of myosin. The validity of the entropy-electrostatic principle is discussed in the explanation of the mechanism of contraction as based on the polyampholytic nature of the contractile system. Suggestions are made in regard to the transformation of chemical energy of ATP into mechanical work of contraction. This is conceived as a biphasic process linked with two stages of the enzymatic interaction of ATP with myosin, one being the addition of ATP to the reactive centers of myosin, and the other being the cleavage of ATP.

Poglazov *et al.* (4) have shown that, by means of amperometric titration with silver, it was possible to demonstrate 8.3 to 8.9 $\times$ 10^{-8} moles of thiol groups per mg. of myosin, while the titration with mercuric chloride in borate buffer revealed a lower value. In myosin, only the free and slowly re-

acting groups could be revealed by the amperometric titration, and the masked thiol groups could not be detected. By use of a modified nitroprusside reaction and suitable corrections, 2.1 to 2.5×10^{-8} moles per mg. of myosin of free thiol groups were found. By means of the nitroprusside reaction, an interaction between the free thiol groups of myosin and ATP was indicated. Amperometric titration, however, failed to reveal such interaction. The authors show that 2×10^{-5} moles ATP binds about 70 per cent of the thiol groups revealed by the nitroprusside reaction. This interaction appears to be specific, suggesting enzyme-substrate relationship. The stabilizing effect of ATP during heat treatment of myosin extends to the ATPase activity of myosin, to the formation of actomyosin complex, and to the viscosity of the myosin solution. It appears that the thiol groups of myosin participate in the stabilizing effect of ATP.

Carbohydrate metabolism and enzymes.—Gershanovich et al. (5) observed that kidney tissue cultivated in tissue cultures acquired in a short time metabolic characteristics which were not present in the original kidney tissue, namely, an intense aerobic and anerobic glycolysis, apparent damage to the systems which oxidize pyruvate and p-phenylenediamine, and the reverse Pasteur effect. The characteristics observed in the kidney tissue cultures approach those of a malignant tissue. The authors ask whether the observed changes in the explanted tissue are indications of malignancy. It is known that repeated transfer of cells is generally connected with production of tumors, that anerobic conditions for growth favor malignization, or that exposure to carcinogens elicits malignancy. In the present experiments, none of these factors was operating during the 7 to 12 days of cultivation of the kidney tissue. The observed high rate of the hexokinase reaction, accompanied by a high rate of aerobic glycolysis with the production of lactic acid, and a high rate of aldolase reaction observed by Levy & Baron (6) in kidney tissue grown *in vitro* do not fit well into the concept of Warburg (7, 7a), which states that a high level of aerobic glycolysis is a specific characteristic of malignant tissue. Evidence is available to show that the characteristics ascribed to malignant tissue exist in normal tissues as well. Of some interest is the elucidation of these characteristics of metabolism, since they are essential for the multiplication of virus. Thus, it is known that, during the latent period of poliomyelitis virus multiplication, there occurs an activation of glycolysis in the kidney tissue medium (6). It is also known that respiration is essential for the multiplication of the virus and that the polio virus could be cultivated on neoplastic tissue as well as on monkey kidney. The significance of the observed changes in the kidney cultures for the synthesis of virus protein is a problem for future studies.

Šlechta et al. (8) observed an inhibition of glycolysis and fructolysis by 2-deoxyglucose in Ehrlich ascites cells. The data show that 2-deoxyglucose competitively inhibited the activity of hexokinase which was located in the cell wall and which phosphorylates glucose when the latter passes through the wall. Fructose, in contrast to glucose, penetrates the cell wall by simple

diffusion. 2-Deoxyglucose is phosphorylated by the intracellular hexokinase. Presumably, fructose is phosphorylated also by the intracellular hexokinase, and its phosphorylation is completely inhibited by 2-deoxyglucose inside the cell. It would appear that the hexokinase localized inside the cell phosphorylates only fructose, the hexokinase localized in the cell wall phosphorylates glucose, and the two hexokinases are substrate specific.

Elzina & Engelhardt (9) reported that the specific activity of lactic acid produced on incubation of Ehrlich ascites cells with glucose-1-C^{14} and with glucose-6-C^{14} is about the same, suggesting that about 90 to 95 per cent of glucose was catabolized by the cells via the Embden-Meyerhof pathway, if one employs the assumptions and the calculations of previous investigators (10). Under aerobic conditions, the radioactivity of proteins of the ascites cells in incubation with glucose-1-C^{14} and with glucose-6-C^{14} was also about the same. Under anerobic conditions, the activity of proteins decreased about tenfold. It is suggested that the carbon atoms of glucose enter cell proteins via glycolysis to lactic acid and during aerobic conditions, via the formation of an active three-carbon compound by dehydrogenation, amination, and entry into the tricarboxylic acid cycle. The extent of radioactivity of RNA of the ascites cells, incubated with glucose-1-C^{14} under aerobic conditions, was twice that obtained on incubation with glucose-6-C^{14}. The isolated pentose of the RNA confirmed the data obtained on intact RNA. This suggests that the basic route of the formation of pentose of RNA in tumor tissue is not the hexosemonophosphate shunt, but reactions involved in the transketolase-transaldolase pathway.

Neifakh & Fomina (11), in a kinetic study of the hexokinase (HK) isolated from rat skeletal muscle and from rat rhabdomyoblastoma, found that for glucose the Michaelis constant (K_m) of the tumor hexokinase is 5×10^{-4} M and for the muscle hexokinase the K_m is 2×10^{-3} M i.e., that the tumor hexokinase has a much greater affinity for glucose than the muscle hexokinase. The determination of the activity of muscle hexokinase showed that the Q_{HK} is 3.88 ± 0.61, while the Q_{HK} of the muscle hexokinase was only one-fourth of that of 6-phosphofructokinase and about 1000 times lower that the Q of phosphohexoisomerase. Compared to muscle hexokinase, the tumor hexokinase is more active (Q_{HK} is 6.72 ± 0.91), although less so than the other glycolytic enzymes. It is suggested that with glucose as the substrate the maximum rate of glycolysis in tissues depends on the hexokinase activity, which is the "slowest enzymatic link," whereas with glycogen as the substrate the rate of glycolysis is determined by the activity of 6-phosphofructokinase. It is also suggested that the high level of glycolysis in tumors results from a greater affinity for glucose and to a more powerful activity of tumor hexokinase.

Neifakh & Melnikova (12) studied further the enzymatic links which determine the rate of glycolysis in muscle. They assumed that only that enzyme limits the rate of glycolysis which will increase the rate of reaction upon its addition, or activation, to a complete enzymatic system. Such a

complete enzymatic system employed was as follows: dialyzed muscle extract, prepared according to Meyerhof, was supplemented with (in moles per liter) ATP and ADP, 10^{-3}; DPN, 2.5×10^{-4}; $MgSO_4$, 3×10^{-8}; nicotinamide, 5×10^{-8}; fructose-6-phosphate, 1.1×10^{-4}; cysteine, 2×10^{-3}; glycogen, 0.3 per cent, or glucose, 1.1×10^{-2}; fructose-1,6-diphosphate, 1×10^{-4}, in a final volume of 2.5 ml. In micromoles of lactic acid per gram of tissue per hour, the maximum rate of glycolysis *in situ* is 1570 (13). In the *in vitro* system described above, the maximum rate of glycolysis was 1300 to 2680. The addition of fructose phosphokinase to preparations containing glucose did not increase the rate of glycolysis. The addition of fructose phosphokinase to preparations containing glycogen or fructose-6-phosphate increased the glycolysis rate six- to eightfold, and this increase was proportional to the amount of fructose phosphokinase added in the presence of maximum amounts of either glycogen or fructose-6-phosphate, i.e., the acceleration effect of fructose phosphokinase followed the general kinetic rules of enzymatic reactions. Fructose phosphokinase was prepared according to Ling, Byrne & Lardy (14). Aldolase, 3-phosphodehydrogenase, or lactic dehydrogenase, were inactive in catalyzing the glycolysis in muscle, although these enzymes were proposed by others as the "slowest" in the glycolytic path. Hexokinase had no effect on the breakdown of fructose-6-phosphate to lactate. However, the same enzyme, in similar amounts, increased the rate of breakdown of glucose to lactic acid ten- to fourteenfold. The authors reason that the fact that hexosemonophosphate is present in tissue in isolable amounts, while fructose-1,6-diphosphate is present in tissue only in traces, indicates that the limitation of glycolysis begins at a stage preceding the formation of fructose-1,6-diphosphate. These critical steps are the activities of fructose phosphokinase and hexokinase. In those tissues in which the predominant substrate of glycolysis is glycogen, such as the muscle, the maximum rate of reaction is limited by fructose phosphokinase. In other tissues, in which the principal substrate is glucose (nerve tissue, heart, erythrocytes, and malignant tumors), the maximum rate of glycolysis is limited by the activity of hexokinase. The fact that the synthesis of glycogen in muscle proceeds with the participation of hexokinase does not contradict this hypothesis, as it is known that the synthesis of glycogen and its degradation are separate reactions in time, taking place during various phases of muscular contraction and at different rates.

Luganova, Seitz & Teodorovich (15) found that human leucocytes and leucocytes of patients with myeloid leucosis show capacity for aerobic glycolysis (60 per cent of that observed under anaerobic conditions). Lymphocytes did not form lactic acid under aerobic conditions, but under anaerobic conditions, the rate of glycolysis in lymphocytes was comparable to that shown by the granulocytes. In some patients with leucosis, leucocytes were found which did not glycolyze glucose under either aerobic or anaerobic conditions. These leucocytes degraded their own glycogen to lactic acid independently of the extent of aeration. Leucocytes of normal humans and those

of patients with leucosis showed a reverse Pasteur effect (inhibition of respiration by 30 per cent on addition of glucose). In lymphocytes, glucose did not inhibit respiration. Apparently, glucose inhibits respiration only in those cells in which aerobic glycolysis proceeds. In all types of leucocytes examined, the rate of resynthesis of ATP under anaerobic conditions was comparable to that under aerobic ones. The existence of aerobic glycolysis, of the reverse Pasteur effect, and of the capacity to synthesize ATP under aerobic and anaerobic conditions in leucocytes suggests a similarity in the metabolism of leucocytes and malignant cells. These similarities once more indicate the unspecific nature of these metabolic indicators as far as malignancy is concerned, and they emphasize the absence of causal relationship between these metabolic peculiarities and malignancy.

The same authors (16) observed a 20 per cent inhibition of respiration by addition of glucose to fresh thrombocytes. Under anaerobic conditions, 1 ml. of platelets formed 9.7 mg. of lactic acid per hour ($Q_{CO_2}^{N_2} = 19.3$), and under aerobic conditions, 6.3 mg. ($Q_{CO_2}^{air} = 12.0$). One ml. of thrombocytes contained 150 μg. of labile ATP phosphorus and 57 μg. of phospholipide P. No DNA was detected in thrombocytes. Under anaerobic incubation of thrombocytes with glucose, the ATP content remained the same. After 1 hr. of incubation with glucose, the rate of renewal of ATP phosphorus or that of RNA of thrombocytes was about the same under anaerobic or aerobic conditions. The C^{14} of the uniformly labeled glucose entered the proteins and the RNA of blood platelets. The metabolic coefficients obtained on the thrombocytes are close to those characteristics which Warburg considers as specific for the cancer cell. The value for the ratio of

$$Q_{CO_2}^{N_2} \text{ to } Q_{O_2}$$

for blood platelets (5.4) is similar to that obtained for the leucocytes (17). These considerations reflect serious doubt about the specificity of the cancer cell in exhibiting quantitative ratios of respiration and glycolysis peculiar to it, as was emphasized by Warburg. The nonspecificity of the cancer cell in this respect is further indicated by the presence of aerobic glycolysis and of the reverse Pasteur effect in leucocytes and thrombocytes and by the ability of thrombocytes to maintain the resynthesis of ATP under aerobic and anaerobic conditions, a property which has been previously observed in the cancer cell (17). That all the described reactions observed in thrombocytes are peculiar to complete cells suggests that a structural organization analogous to that of complete cells exists in thrombocytes, in spite of the absence of the nucleus in thrombocytes.

Stepanenko & Bobrova (18) find that the previously observed instability of the sodium salt of fructose-1,6-diphosphate was caused by bacterial contamination of the product. Stable, sterile preparations of the salt are described. These preparations were found to have a stimulating activity on the heart, particularly after surgical shock. On further purification of the product by chromatography, a compound, arbitrarily called "ZSC," was isolated

from the preparations. Thus far, uracil, pentose, and phosphoric acid were identified as components, and this strongly suggests that the "ZSC" is a uridine phosphate derivative. The strong biological effects of the preparation of fructose-1,6-diphosphate were attributed to the synergistic effects of "ZSC" contaminating the fructose-1,6-diphosphate preparation.

Leites, Rabkina & Smirnova (19) report that a single injection of 25 to 50 mg. of $CoCl_2$ to rabbits induced hyperglycemia within the first 3 to 5 hr. Hyperplasia and hypertrophy of the α-cells of the islet tissue were also noted, and these conditions remained after the return of blood sugar to normal levels. Administration of $CoCl_2$ to rabbits did not alter the liver glycogen content, but it altered the latent alloxan diabetes in rabbits into an obvious one when hyperplasia of the α-cells was present. The data suggest that hyperplasia and hypertrophy of the α-cells are not connected with increased production of glucagon by the α-cells and that the changes in the glycemic state could have resulted from a decreased secretion of insulin as a consequence of a relative decrease in β-cells. The hypoglycemic effects induced by carbutamide (synonyms: Nadisan, BZ-55.) are not associated with any morphological changes in the pancreatic apparatus. Leites & Smirnova (20) also found that carbutamide inhibited *in vivo* and *in vitro* the activity of insulinase of the liver of normal and diabetic animals and that the drug accentuated the hypoglycemic effect of insulin in normal and in alloxan diabetic or depancreatized animals. In completely depancreatized dogs and rabbits with severe alloxan diabetes, carbutamide did not elicit a hypoglycemic effect. In mild alloxan diabetes (part of the pancreas is intact) carbutamide induced hypoglycemia. It is concluded that the mechanism of the action of carbutamide is via the inactivation of insulinase. Karaev *et al.* (21) report that the extracts of *Cichorium intybus, Lactuca sativa, Coriandrum sativum, Aloe arborescens,* and *Schizandra chinensis* markedly improved the utilization of glucose by rabbits, as was indicated by the glucose tolerance tests, and suggest that the presence of active ingredients in these plant extracts might be of use in the treatment of diabetes in man.

Solomatina (22) administered P^{32} to alloxan diabetic male rats; muscle and liver ADP and ATP were isolated chromatographically and their specific activities determined. In diabetic rats the amount of inorganic phosphate in the TCA filtrates of liver and muscle increased over that found in normal rats by 31 and 35 per cent, respectively. A 60 to 65 per cent increase in the amount of inorganic phosphate in the filtrates of liver and muscle of dinitrophenol-poisoned rats was observed. The relative specific activity of ATP in diabetic livers decreased and that in the muscle increased; similar changes were noted in the DNP-poisoned animals. In diabetic rat liver, the total amounts of ADP and ATP, or of the ratio of ATP/ADP, remained normal, being about 1:1. In the muscle of normal rat, the ATP/ADP ratio is about 5:1. In the muscle of the diabetic rat, however, the ATP/ADP ratio was 2.8:1.

Bekina & Petrova (23) presented comparative data for the determina-

tion of activity of liver hexokinase by a decrease in either the ATP or glucose in the medium. The more reliable procedure, according to the authors, was that based on the determination of the decrease of ATP in the medium which contained NaF and inorganic phosphate to depress the activity of phosphatases. The amount of phosphorylated glucose was found to be about 140 μg. per ml. of the medium which contained liver in 1:20 dilution. This value is considerably higher than hitherto reported for rabbit liver, but it is comparable to the value of 65 to 75 μg. reported by Slein, Cori & Cori (24), if due account of the hydrolysis of glycogen and of the activity of glucose-6-phosphatase is taken into consideration. Petrova (25) reported the isolation from rabbit livers of an enzyme of the nonphosphorylating type which is able to catalyze the formation of glycogenlike material in the presence of α-dextrins and free glucose. Petrova suggests that this enzymatic reaction is of a transglucosylase type, in which dextrins act as the donor-substrate and free glucose as the acceptor co-substrate. Lukomskaya & Rosenfeld (26) find that the spleen, liver, kidney, lungs, brain, and muscle of several animals contain an enzyme which cleaves dextran, named α-1,6-dextranglucosidase, which is thermostable up to 60° at pH 4.8, and labile at neutral pH, with an optimal activity at pH 4.7-5.0. The activity of this enzyme was suppressed by glucose, isomaltose, and maltose. Shemanova & Blagoveshchensky (27) detected the activity of fructosediphosphate aldolase in *Clostridium oedemantiens*. Its activity was inhibited by Ca^{++}, Mg^{++}, Mn^{++}, and Zn^{++}. On incubation with glucose, the following compounds were identified in the medium: glucose-1-phosphate, glucose-6-phosphate, fructose-6-phosphate, fructose-1,6-diphosphate, phosphopyruvic and lactic acids.

Sherstnev (28) finds that the reported increased oxygen consumption and esterification of inorganic P in muscle tissue of normal and tumor-bearing rats on addition of carnosine to the preparations can be reproduced by the addition of either arginine or lysine. The effect is attributable to the rise in pH of the medium and not to any specific role of carnosine in the consumption of O_2 or esterification of inorganic P. Chetverikova (29) reports that the administration of Medinal to rabbits increased the rate of reduction of methylene blue at the expense of endogenous substrates in the liver, increasing at the same time the activity of liver succinic dehydrogenase. *In vitro*, Medinal increased the activity of liver succinic, isocitric, and lactic dehydrogenases; higher concentrations of the drug inhibited the activities. The inhibition of respiration of liver slices obtained from animals under Medinal anesthesia could be reproduced *in vitro* with low or high concentrations of the drug. Ryumina *et al.* (30) found that 24 hr. after hourly administration for 3 hr. of picric acid, methylene blue, or phenylhydrazine to rabbits, the resulting hemolytic anemia was accompanied by 150 per cent rise in glycemia and by a decrease in glycolysis to lactic acid, as a result of the degeneration of the erythrocytes induced by the hemolytic agents. Kiverin (31) concluded that the principal path of carbo-

hydrate breakdown in rabbit skin and that of guinea pigs is amylolytic, and not phosphorolytic. Melnikova & Surikova (32) reported that *Penicillium chrysogenum* produces oxalic acid during the synthesis of penicillin in agitated cultures. The addition of sugars to the acetate-lactate medium increased the production of oxalic acid, and the addition of phenylacetic acid or of its amide inhibited the production of oxalic acid from the sugars, presumably by increasing their oxidation. The accumulation of oxalic acid by the mold, lowering the pH of the medium, decreased the production of penicillin by the mold. Vyskrebenzeva (33) finds that the coelomic fluid of the silkworm, *Bombyx mori,* is capable of performing many reactions which accompany tissue respiration. Thus sucrose was metabolized in the fluid to phosphate esters and organic acids, which were in part employed for the synthesis of amino acids. Glycolytic poisons inhibited the synthesis of organic acids, such as pyruvic acid, from sucrose. The analysis of the phosphate esters revealed them to be phosphorylated polyatomic alcohols of unknown nature. The usual phosphate esters of glycolysis and of the pentose cycle were absent. On incubation of sucrose-C^{14} with the coelomic fluid, the C^{14} was located in malic, fumaric, and succinic acids, suggesting that the transformations of pyruvate are basically connected with the synthesis of dicarboxylic acids from pyruvate by carboxylation. The synthesis of citric acid proceeded at an insignificant rate. The latter is formed not from sugars but from organic acids which arise in the course of degradation of fats. These observations, the author concludes, suggest that the anaerobic oxidation of carbohydrates predominates in the coelomic fluid of the silkworm.

Elpiner & Sokolskaya (34) subjected solutions of arabinose, glucose, sucrose, maltose, and raffinose to irradiation by ultrasonic waves of frequency of 385 kc./s and 4 to 5 watts/cm.^{2} intensity at 25 to 30°, and the material in the solutions was examined spectrophotometrically in ultraviolet before and after the irradiation. All the sugars employed gave rise to compounds with absorption maximum at 265 mμ, the extent of absorption at 265 mμ increasing with time of irradiation. After the irradiation, the solutions of sucrose and raffinose showed the presence of reducing sugars in either acid or alkaline pH. Mannite or glycerol also gave rise to reducing substances with absorption maximum at 265 mμ. On acidification of the irradiated alkaline solutions of sugars and complex alcohols to pH 2, the absorption maximum shifted from 265 mμ to 245 mμ. Organic acids with conjugated double bonds, ascorbic acid being one of these, give the absorption maximum at 245 mμ in acid and 265 mμ in alkaline pH. Ultrasonic waves break the oxygen bridges in sucrose and raffinose, releasing the aldehyde groups; the process is pH-independent. On ultrasonic irradiation at alkaline pH, the enol form of the aldehyde predominates. Kuzin & Saenko (35) have shown that the green leaves of Tradescantia fix CO_2-C^{14} in compounds which are permeable to collodion membranes, ruling out proteins

as the CO_2 acceptors. The C^{14} appeared in the first 5 sec. of photosynthesis in compounds of different Rf and salt solubilities, with particularly high C^{14} concentration in the fraction which contained uronic acids, thus confirming the earlier reports of others (36) and of Calvin that uronic acids are probably the primary products of photosynthesis. Petrova & Bekina (37) reviewed the available information on the alteration of the processes of carbohydrate-phosphorus metabolism during diabetes.

Proteins, amino acids, and enzymes.—In January of 1958, a Conference on the Problems of Proteins was held in Moscow in which various aspects of protein synthesis were discussed by several participants from the Soviet Union and adjacent countries. At the conference it was concluded that the Soviet Union lacks adequate facilities and specialized laboratories for the study of protein structure and that the existing system of education of scientific workers does not guarantee the implementation of biochemists with the necessary knowledge of physics, mathematics, and physical chemistry, subjects which are essential for the successful study of protein structure (38). By the action of the Academy of Sciences of the U.S.S.R. in 1958, a National Committee of Soviet biologists was organized, with V. N. Sukachev as the chairman of the Committee. The Committee was charged with the responsibility for the dissemination of information about the work of Soviet biologists, provision of measures for the facilitation of participation of Soviet biologists in National and International meetings, attraction of foreign scientists to biological conferences held in the U.S.S.R., and facilitation of exchange of books between foreign and Soviet biologists. The address of the Committee is Leninskii Prospect, 37, Moscow B-71, U.S.S.R. (39).

Krizman *et al.* (40) continued their studies on the "incorporation" of free amino acids into the isolated proteins (myosin and blood serum proteins) in phosphate buffer, pH 7.5, at 37°. A linear relationship was observed between the extent of "incorporation" of glycine, tyrosine, and methionine and the concentration of the amino acids in the medium within a wide range. The rate of "incorporation" of cysteine and methionine into myosin was stimulated by ATP. Shnol (41) observed that the extent of "incorporation" of methionine, glycine, or tyrosine into egg albumin *in vitro* was linear with time, the maximum being reached at 0.2 to 0.3 per cent "incorporation." Peptide bonds were found to be formed with terminal amino or carboxyl groups of the protein, and, according to Shnol, this observation can hardly be considered as evidence for "protein synthesis." Binding of the labeled amino acids by the protein was not accompanied by a release of equivalent amount of amino acids from the protein, and the rate of "incorporation" of labeled amino acids was not affected by dilution of the labeled amino acids with unlabeled amino acids. Calculations showed that each 50 to 100 molecules of the protein bound one molecule of the amino acid at the point of equilibrium.

Chi Cheng Wu & Orekhovich (42) prepared a highly active acylase I from the acetone powder of hog kidney, and the activity of this preparation was five to nine times that described by Greenstein and co-workers (43, 43a). The purified acylase I showed greater specificity toward acetylalanine and chloracetylalanine, and at pH 3 to 5 it possessed the activity of cathepsin. Twenty mg. of hemoglobin and 2 mg. of acylase I in 4 ml. of acetate buffer (0.1 M) at pH 4 and 37° cleaved 40 per cent of the protein. Lokshina & Orekhovich (44) found that in the course of activation of dinitrophenyl (DNP)-pepsinogen the amount of N-terminal DNP-leucine in the protein decreased, indicating that the peptide or peptides, formed in the course of activation of pepsinogen are split off in the N-terminal region of the zymogen molecule. Upon additional dinitrophenylation of the activated protein, the amount of N-terminal DNP-leucine increased, as compared with the untreated protein. Thus, the N-terminal pepsin residue—leucine—is liberated in the course of activation of pepsinogen as a result of hydrolysis of the peptide bond which binds the amino group of leucine. However, on the chromatogram it was impossible to differentiate the DNP derivatives of leucine and isoleucine, and the possibility is not excluded, therefore, that in the course of hydrolysis the peptide bond which binds isoleucine is also involved. Orekhovich & Shpikiter (45) found the sedimentation constant for the α-component of procollagen to be s $= 4$ S, the diffusion constant, $D = 2.6 \times 10^{-7}$ cm.^{2}/sec., and the molecular weight, $M = 125,000$. For the β-component of procollagen, s $= 5.7$ S; $D = 1.6 \times 10^{-7}$ cm.^{2}/sec., and $M = 290,000$. The ultracentrifuge method showed that the weight ratio of α- and β-components of procollagen was 1:1, suggesting that the procollagen molecule contains two parts of the α-component and one part of the β-component. Millionova & Andreeva (46) reported that the x-ray analysis of collagen revealed the presence of a specific polypeptide chain configuration in the protein, consisting of imino acid and glycine residues. The imino acid to glycine residue ratio was found to be 1:1. In a lecture in the United States, Orekhovich & Shpikiter (47) reviewed their own work and the data of others on procollagens.

Kretovich, Smirnova & Frenkel (48) showed that glycinine obtained from soybean cotyledons in a 10 per cent NaCl solution consists of two independently sedimenting components with sedimentation constants (s) of 14.0 and 8.1 Svedberg units and molecular weights of 330,000 and 126,000, respectively. The same authors (49) now report that, on solution of glycinine in a cysteine solution, a third component appears with the sedimentation constant of 10.7 S and of molecular weight of 245,000. On addition of 10 per cent NaCl to this solution, the protein reverts to its original state. It has been pointed out by Naismith (50) that glycinine exhibits a reversible association of its components in the presence of low ionic concentrations. Kretovich et al. (49) now show that the "individual components" of glycinine can be transformed into one another, depending on the condition

of dissolution. In other words, the "individual components" of glycinine are oligomers of the same basic submolecule, probably the S_8 component of the molecular weight of 128,000. During association, the most stable form is the S_{14} component. It appears that, depending on the conditions of dissolution, there is a slow attainment of equilibrium at which there is a separation into the individual components. Polyvalent ions, such as cysteine and ATP, substantially alter this equilibrium. The solubility effects of cysteine are reflected in the increase in the number of individual components in solution. The possible mechanism of action of the polyvalent ions consists in binding of the ions by the macromlecules of the protein, thereby altering its effective charge and modifying the electrostatic interaction between the globules which determine the equilibrium between the monomeric and the oligomeric forms.

Kretovich & Uspenskaya (51) reported that the homogenates of pea seedlings synthesize phenylalanine from phenylpyruvic acid by transamination with glutamic acid, aspartic acid, and also, at slower rates, with serine, valine, and leucine. Hydroxylamine inhibited the reaction.

Firfarova (52) determined the amino acid content, by the method of Levy (53) as modified by Braunitzer (54), of α-crystalline which was prepared by the previously described procedure of Orekhovich et al. (55). The amino acid composition (in per cent) was as follows: aspartic acid, 5.5; glutamic acid, 17.0; threonine, 2.8; serine, 5.5; valine, 5.4; alanine, 3.1; leucine and isoleucine, 20.0; glycine, 3.5; methionine, 1.7; phenylalanine, 11.2; proline, 5.2; lysine, 4.8; cysteine, 0.3; arginine, 6.7; histidine, 7.5; tyrosine, 7.5; and tryptophane, 1.3. N-Terminal amino acid was glutamic acid (2 residues per mole of protein), and C-terminal amino acid was alanine.

Semenov (56) described an apparatus for desalting amino acid solutions, which, according to the author, is superior to that described by Consden, Gordon & Martin (57) in that it permits the use of about 1 to 3 ml. of amino acid solution in about 5 to 7 min. The greater rate of desalting results from the greater concentration of the solution employed as well as to the prevention of reverse diffusion of ions and entry of water into the solution.

Khesin et al. (58) reported that, upon incubation of cytoplasmic granules of pancreatic cells of pigeon in Krebs-Ringer bicarbonate buffer in the presence of all L-amino acids, the protein content increased by 4.3 per cent, while the total amount of synthesized protein was 9.1 per cent (after correction for autolysis). The components of large granules of pigeon pancreas and of rat liver are heterogeneous, consisting of mitochondria and lighter granules which are rich in RNA. Protein synthesis takes place in the lighter granules, continuing for 15 to 20 min.; thereafter decomposition of protein predominates over the synthesis. The synthesis of protein occurs only in the presence of all amino acids, essential and nonessential ones.

Deficiency in a single amino acid stops or retards the synthesis of protein. The cytoplasmic granules synthesize protein only in the medium in which mitochondria have been previously incubated with the amino acids, in the presence of ATP and an aerobic respiratory substrate. The compounds which are essential to protein synthesis from amino acids in other granules, including microsomes, are formed in the mitochondria in the presence of ATP. These are not labile phosphorus compounds, since their activity is not affected by heat at 100° in 1 N HCl for 7 to 20 min., and since they are not replaceable by ATP. After preincubation with mitochondria of amino acids, in the presence of ATP, the cytoplasmic granules are able to synthesize protein in the absence of ATP. This synthesis occurs also if disintegrated cytoplasmic granules are employed and if the stipulated conditions of pre-incubation with mitochondria and ATP are observed.

Sissakiyan & Kuvaeva (59) reported that in the body fluid of the silk-worm, *B. mori,* Ukrainian strain No. 1, histogenesis is accompanied by an intense protein synthesis, particularly on addition of 19 amino acids to the fluid. The addition of only the essential amino acids yields better results, which was explained as an indication of the body fluid capacity to syn-thesize all the nonessential amino acids required for the protein formation. The observed need for a prolonged incubation of the fluid with the amino acids was assumed to be caused by the stepwise nature of the amino acid build-up into the insect protein. A peptide containing nine amino acids (arginine, histidine, aspartic acid, serine, glycine, glutamic acid, threonine, alanine, and tyrosine), 1.27 per cent P, and 2 per cent glucose, was isolated from the insect fluid, which, when incubated with radioactive glycine, in-corporated the amino acid in increasing amounts with the increase of time of incubation. The extent of incorporation of radioactive glycine into the isolated peptide paralleled the extent of incorporation of radioactive gly-cine into the insect fluid protein.

Blanchard *et al.* (60) suggested that the role of biotin in the biosyn-thesis of dicarboxylic keto acids and amino acids and in other enzymatic reactions is connected with the participation of biotin in the synthesis of enzyme proteins not in the synthesis of cofactors. Poznanskaya (61), in Braunshtein's laboratory, has now shown that the synthesis of the enzy-matically active protein, pancreatic amylase, and of the protein, devoid of enzymatic activity, serum albumin, is greatly reduced in biotin-deficient chicken tissues (pancreas and liver slices, respectively, were used). The apparently specific nature of this inhibition was further revealed by the restoration of the synthesis of the respective proteins in the pancreas and liver slices on injection of 100 μg. of biotin into the deficient chicks. How-ever, the addition to slices of pancreas of biotin-deficient chicks of α-keto-glutaric acid completely restored the capacity of the tissue to synthesize amylase (fumarate was only partially effective). Similar restorative effect on the synthesis of serum albumin in biotin-deficient liver slices was pro-

duced by glutamine and asparagine, which readily penetrated the liver cells and there were transformed into the respective keto acids. According to Poznanskaya (61), the data strongly indicate that biotin per se is not involved in the direct synthesis of proteins and that the inhibition of protein synthesis by biotin deficiency is a reflection of an alteration in the mechanism of the tricarboxylic acid cycle. The compensation of this alteration in the tricarboxylic acid cycle by the ketoglutarate and its precursors (fumarate, glutamine, asparagine) is explained by the restoration by the ketoglutaric acid in the deficient tissues of oxidative phosphorylation, i.e., the formation of ATP, which is necessary as the energy source for the synthesis of protein. Furthermore, the added dicarboxylic acids serve in the tissues as the precursor material for the synthesis of a number of non-essential amino acids, which are utilized in the synthesis of protein (in the experiments only the essential amino acids were used). A similar inhibition of amylase synthesis in slices of normal pancreas was induced *in vitro* by the inhibitors of the citric acid cycle, mesotartaric acid (inhibition of carboxylation of pyruvate) or fluoroacetate (inhibition of aconitase). According to Poznanskaya (62), the increase in the activity of tryptophan peroxidase in rat liver, induced by tryptophan administration to rats, is not affected by deficiency in biotin. The author suggests that the adaptive increase in the activity of tryptophan peroxidase in the liver of mammals is not associated with the *de novo* synthesis of the enzyme protein. Shtraub (63) and co-workers found that the liver of mammals contains a large quantity of inactive tryptophan peroxidase. On electrophoresis of the liver extracts on paper, the activity of tryptophan peroxidase was increased thirty- to fortyfold. Such a demasking of enzyme activity accounts well for the increase in activity of tryptophan peroxidase by tryptophan induction, which generally is of the order of seven- to tenfold. Velikodvorovskaya (64) reports that, in rats treated with CCl_4, the activity of tryptophan peroxidase and of kynureninase of the liver is impaired, and the activity of tryptophan peroxidase in the CCl_4-treated rats can be markedly increased by the administration of tryptophan or by the addition of tryptophan to the CCl_4-damaged liver slices, suggesting that the capacity for the "adaptation" of tryptophan peroxidase during CCl_4 poisoning was fully preserved *in vivo* and *in vitro*. In CCl_4-damaged rats, the amounts of excreted xanthurenic acid and of N'-methylnicotinamide was greater than in normal rats. $CHCl_3$ had no effect on the activities of liver tryptophan peroxidase or kynureninase. In connection with the induction of tryptophan peroxidase activity by tryptophan, it is of interest to mention here the report by Nemeth & Nachmias (65) that the relative refractoriness of fetal liver to tryptophan suggests that substrate induction is not the rate-limiting mechanism which controls the changes in tryptophan peroxidase activity during development, as has been suggested by Knox (66) to be so for the adult liver.

Braunshtein & Azarkh (67) reported that fluorocitrate inhibited the

formation of alanine from pyruvate and ammonia by rat liver homogenates in 0.1 M phosphate in the presence of EDTA. Neither was there any formation of glutamic acid from ketoglutarate and ammonia under these conditions. The inhibition of alanine formation by fluoroacetate was reversed by citrate and ketoglutarate, with the simultaneous decrease in the formation of glutamate. The results were interpreted as confirmation of the synthesis of the amino acids via transamination from glutamate.

Oparin, Gelman & Deborin (68) reported that protoplasts of *Micrococcus lysodeikticus,* incubated with a mixture of 19 amino acids, synthesize protein if the concentration of sucrose in the medium is > 0.44 M. This increase in protein was not inhibited by biomycin or 2,4-dinitrophenol, although these substances inhibited the incorporation of glycine into the protein. Trypsin and ergosterol formed a complex which, from surface pressure measurements, was found to contain two molecules of trypsin. This complex was found to have a slightly higher enzymatic activity toward serum albumin than trypsin.

Kowalski *et al.* (69) observed that human tissue contains a substance which, after activation by streptokinase, shows fibrinolytic activity. It was suggested that the tissue proenzyme is identical with blood plasminogen or that it is one of the cathepsins capable of activation by streptokinase. It would appear that tissues contain a plasminogen which is identical with that of plasma. Golovanova (70) established the presence of fibrinogenase in fresh urine of healthy subjects by isolation of the active enzyme by ethanol precipitation and dialysis, followed by freeze-drying. The enzyme solubilized fibrin clots which were obtained by coagulation of fibrinogen with thrombin. The activity of the enzyme was destroyed by heating at 80°. During the action of fibrinogenase on fibrin, there was no appearance of nonprotein nitrogen, even after 24 hr. at 38°. These results are not consonant with the opinion of Macfarlane & Pilling (71) in regard to proteolytic degradation of fibrin by an enzymatic factor present in urine. The results rather suggest the identification of the "factor in urine" with fibrinogenase of blood plasma, which, however, while in plasma, is inactive. The active fibrinogenase, as found in urine, is released through the kidneys without the inhibitor, which remains bound to the albumin fraction of plasma.

Chaplygina (72) studied the effects of the intravenous administration to guinea pigs of "active globulins" isolated from human plasma. Dry preparations, dissolved in water, were administered intracardially. Five to 10 min. after the injection of fibrinogenase, the blood lost its capacity to coagulate. The addition of normal blood, however, to the blood of animals which received fibrinogenase induced coagulation, while the addition of of thrombin did not. The clot which was formed on addition of normal blood to the blood of fibrinogenase-treated animals soon dissolved, suggesting that the hemolytic blood acquired fibrinolytic activity. It was further emphasized that the introduction of fibrinogenase into the blood stream of

guinea pigs induces hemophilia. The rapid reappearance of fibrinogen in blood after its complete disappearance under the influence of fibrinogenase was seen as a reflection of the compensatory capacity of the liver, producing fibrinogen, and the rapid disappearance of fibrinogenase activity from the blood (10 to 15 min. after its introduction into the blood stream) as possibly a reflection of the rapid binding of the fibrinogenase by the inhibitor and of the excretion of fibrinogenase in the urine. Il'in *et al.* (73) suggested that the development of the activation of fibrinogenase in blood of animals is under the control or influence of the higher nervous system. Injection of epinephrine to cats, these authors reported, induced the activation of blood fibrinogenase in 13 out of 16 experiments. Kudryashev & Ulytina (74) discussed the existence and significance of a physiological anticoagulating system.

Balandin (75) reported that the principles of structure and energy correspondence of the multiplet theory of catalysis can explain the high activity and specificity of the enzyme action. The enzymatic reactions fit into the classification of the multiplet theory as reactions of the duplet and triplet type. The principle of structure correspondence applied to the groups of reacting atoms requires a definite relationship to be fulfilled between the interatomic distances in the reacting groups and those in the parts of the enzyme surface which are in mutual contact. The same principle is also applicable to the substituents attached to the reacting groups. Therefore, the substituents should be superficially isoamorphous with the parts of the apoenzyme surface adsorbing them. The principle of energy correspondence manifests itself in three ways: (*a*) there should be fulfilled a definite relationship between the bond energies within the reacting group of the substrate and those of the atoms of the substrate which react with the atoms of the enzyme molecule; (*b*) the substituents influence the reaction velocity because they influence the magnitude of the bond energies indicated above, the influence being similar to the auxochromic effect; and (*c*) the juxtaposition of the substituents and atoms of the enzyme surface results in the diminution of the distances between them and, hence, in an increase in the adsorption energy of the substrate molecule, which leads to a lowering of the energy barrier and to a considerable increase in the velocity of the enzymatic reaction.

Grechko (76) described two procedures for the synthesis of β-alanine labeled with C^{14} in the carboxyl and with N^{15} in the amino group. In experiments on intact rabbits and young rats, it was shown that β-alanine per se enters tissue carnosine without any alteration in the C^{14}/N^{15} ratio of the administered doubly labeled β-alanine. The results were consonant with the data of Martignoni & Winnick (77), who employed β-alanine labeled with C^{14} in the carboxyl group.

Kritsky (78) reported that the extent of incorporation of glycine-1-C^{14} *in vitro* into liver hypoxanthine of pigeons exposed to x-rays (2000 r) is

decreased. The extent of incorporation of the C^{14} into the liver malic, suc-
cinic, and fumaric acids also was inhibited in the x-ray-treated pigeon
livers. The disturbance in purine biosynthesis following irradiation is, ac-
cording to the author, connected with the disturbance in the utilization of
the intermediates which are concerned in this biosynthesis, namely, formate,
glutamine, and the organic acids. Filipova & Seitz (79) reported that five
to seven days after the x-ray irradiation of pigeons with a dose of 2000 to
3000 r, symptoms of irradiation sickness appear, followed by death on the
eighth to ninth day. Employing the procedure of Kaplan-Lippman for the
determination of the acetylating capacity of the extracts of the acetone
powders of pigeon liver (sulfanilamide, p-aminobenzoic acid, p-aminoben-
zene, and 4-amino-1,1-azobenzene-4'-sulfonate as the substrates), it was
shown that the irradiation markedly reduced the acetylating capacity of
the liver. The addition of coenzyme A to the irradiated liver preparations
increased the acetylating capacity of the liver, but not to the level observed
with normal preparations, suggesting a possible damage by the irradiation
to the apoenzyme of the system. Measurement of the coenzyme A content
of the irradiated pigeon liver indicated a decrease to about two-thirds of
normal levels. Vezirova (80) found that 14 days after the incorporation of
P^{32} into the soil, the activity of catalase in the leaves of cora and cotton
plants was markedly increased. Relatively low doses of radioactive P and
Fe in the soil also increased the activity of catalase in the leaves of tomato
and egg plants. High dose of P^{32}, and especially of radioactive iron, in the
soil increased the activity of peroxidase in the leaves of corn, tomato, egg
plant, and cotton plant. Pretreatment of the seeds with radioactive P and
Fe also produced plants with leaves of greatly increased activity of catalase
and peroxidase. Mattison (81) reported data to show a close connection
between the biosynthesis of penicillin by *P. chrysogenum* Q-176 and the activ-
ity of peroxidase and catalase in the developing mycelium of the fungus.

Vorobiev (82) obtained X-chymotrypsin, a crystalline form of chymo-
trypsin hitherto unknown, by prolonged activation of α-chymotrypsinogen
with small amounts of trypsin. The product was homogeneous in the ultra-
centrifuge and was similar to α-chymotrypsin in its proteolytic action,
kinetics of proteolysis, capacity to coagulate milk, and in the sedimentation
rate. The author suggests that the chymotrypsinogen B which gives rise to
chymotrypsin B, isolated by Brown *et al.* (83), and its differences from
α-chymotrypsinogen can be explained by the presence in the "chymotrypsino-
gen B" of trypsinogen and of an inhibitor of trypsin. The fact that "chymo-
trypsinogen B" was activated by enterokinase indicates the presence of
trypsinogen in the preparation. The presence of an inhibitor of trypsin in
"chymotrypsinogen B" is indicated by the fact that the maximum value for
the proteolytic activity of "chymotrypsinogen B" was reached only after 10
days of activation, in spite of the large quantity of the added trypsin (the
ratio of trypsin to chymotrypsinogen B was 1:500). Hence, "chymotrypsin-

ogen B" is not an individual protein but a mixture of α-chymotrypsinogen and the above-mentioned contaminants. The crystalline form "chymotrypsinogen B" is, however, similar to that of the isolated X-chymotrypsin, although the activity of the preparations is somewhat different; the variation, however, may result from differences in the methods of estimation of the proteolytic activity. Whether the two proteins are identical or not requires more information.

According to Chernikov (84), the enzymatic hydrolysis of native proteins cannot be explained as cleavage of denatured protein, which is presumed to be always present in equilibrium with the native form. The hypothesis of denaturation and of desegregation effects of proteinases on globulins contradicts the present concept of denaturing process as well as the present information in regard to the mechanism of action of the proteinases. According to all available information, one can postulate that proteinases are able to hydrolyze native globulins, with the exception of certain specific inhibitors of protein nature. Various bonds which stabilize the native state of protein molecules and which participate in the formation of various biological structures, prevent proteolysis. Polypetides with open chains are hydrolyzed by proteinases with greatest speed. Denaturation of globulins increases the rate of enzymatic hydrolysis. Aggregation and renaturation decreases the rate of proteolysis. Thus, the enzymatic hydrolysis of native globulins consists of two stages: one is a genuine hydrolysis, in which the protease hydrolyzes the peptide bonds in the molecule of the original protein or products of its degradation which preserved the structure of the globulins; the second stage is peptidolysis, in which the enzyme hydrolyzes polypeptides which lost the globulin structure. The first stage, the slower reaction, determines the rate of the over-all proteolysis. Mosolov (85) concluded that the theory of specificity of the action of the proteolytic enzymes, developed by the use of low molecular weight synthetic substrates, cannot serve as an adequate explanation of the data obtained with native proteins. During the determination of the nature of the peptide bonds in the protein undergoing enzymatic hydrolysis, important roles are played by the structure and configuration of the molecule of the substrate, the unequal stability of the various peptide bonds, and the concrete conditions under which the enzyme acts.

Berezovskaya (86) has previously shown that the enzymatic system which catalyzes the synthesis of amino acids from pyruvate and ammonia by rat liver is located in the mitochondria. The same author (87) has now prepared an active preparation from rat liver mitochondria and found that addition of liver nuclei to the mitochondrial preparation is required to activate the enzyme to produce the "amino acids."

Liubimova & Fain (88) reported a method for the isolation of a highly active deaminase from myosin. The extracts were obtained from the deaminase preparation by heating in N perchloric acid (89), and the hydrol-

yzates of the preparation in 72 per cent perchloric acid were analyzed (90). The nitrogenous bases thus prepared were analyzed chromatographically. The principal nitrogenous base appears to be cytosine, and a small number of unidentified substances which fluoresced and showed absorption maximum at 250 to 270 mμ were also present. One of these was probably a condensation product of guanine and pentose residues. The main bulk of cytosine was firmly bound to the protein of the deaminase. The phosphoprotein contained P in the ratio of 12 moles of P to one of protein (mol. wt. 3.2×10^5). The nature of the isolated nitrogenous bases of deaminase distinguishes it from myosin and DNA.

Gurvich & Smirnova (91) immunized rabbits with two antigens, horse serum albumin and cat serum γ-globulin, or horse serum albumin and chicken egg albumin. The changes in the content of each antibody were determined, along with the extent of incorporation of glycine-1-C^{14} into the antibodies and into the serum nonspecific γ-globulins. It was found that the incorporation of radioactive glycine into the antibody proteins during the increase in the antibody content proceeded at a much higher rate than the incorporation of radioactive glycine into the nonspecific γ-globulins. This was found to be the more active the more intense was the increas in the antibody content of the blood. The rate of glycine incorporation into the two simultaneously produced antibodies depends on the rate of increase in the content of each antibody, and it is not connected with the immunological specificity. Even a very high rate of formation of one of the antibodies did not inhibit the formation of the other or of non-specific γ-globulins. In fact, during intense neoformation of one antibody, the formation of the other was often stimulated. During the latent period, no appreciable amounts of antibody were formed, nor was there apparently any formation of a precursor protein or of a precursor polypeptide.

Yefimochkina et al. (92) showed that the extent of transamination reaction, studied with N^{15}-labeled glycine, glutamic acid, and ammonium citrate in pyridoxin-deficient rat livers, was essentially the same as in normal livers, indicating a high residual activity of aminopherases in the organs of B_6-deficient rats.

Turpaev (93), on the assumption that acetylcholine exerts its specific action on the effector organs by interacting reversibly with choline receptors, developed the following equation of the kinetics of the reaction between acetylcholine and the effector organ:

$$y = \frac{100 \ (\text{acetylcholine})}{k + (\text{acetylcholine})}$$

where y is the efficiency of acetylcholine action in per cent, and k is a constant. He demonstrated on an isolated heart ventricle of the frog that the efficiency of acetylcholine action is a hyperbolic function of acetylcholine

concentration, which was in accord with the above equation. Exposure of the myocardium to 30 to 40° caused a decrease of the myocardium sensitivity to acetylcholine as a result of a reversible and irreversible heat denaturation of choline receptors. In this case, the efficiency of acetylcholine action, as a function of its concentration, was expressed as follows:

$$y = \frac{(100\text{-}n)\ (\text{acetylcholine})}{k + (\text{acetylcholine})}$$

where n is a relative amount of nonactive choline receptors. The similarity between the equations of acetylcholine interaction with choline receptors and those of the kinetics of enzymatic reactions is attributed to the similarity between the mechanism of complex compound formation of acetylcholine with protein-choline receptors and of the enzymatic reactions between specific substrates and protein-enzymes.

Kowalski et al. (94) showed that various organs of the rat transaminate the amino group of aminolaevulic acid to α-ketoglutaric acid and pyruvic acid. The kidney was the more active organ in this respect, while the red blood cells were practically devoid of activity.

Nucleic acids, nucleoproteins.—Zakharova (95) reported that the regenerated strains of *B. breslau* fall into three groups, according to their adenase activity: those with adenase activity equal to, greater, or smaller than that of the parent strain. With one exception, the guanase activity in all strains was very low. DNA and RNA deaminase activity were also very low. The author concluded that the metabolic pathways of adenine in the parent strain and in the strains regenerated from the culture filtrates of *B. breslau* are dissimilar and that nucleic acids are deaminated at the nucleoside stage. The same author (96) further showed that suspensions of cells of parent strain of *B. breslau* in phosphate buffer deaminated adenine and guanine at the same rate under aerobic or anaerobic conditions. Under aerobic conditions, more of O_2 was consumed than was required for the oxidative deamination. The curve of the O_2 consumption was linear, and this fact suggested that the O_2 consumption was not dependent on the oxidation of the products of deamination of the purines. It was suggested that the purines undergo hydrolytic deamination, thus stimulating the oxidation of other substrates and accelerating the metabolic processes of the cells. A series of strains, obtained from culture filtrates of the parent strain, also deaminated purines, but the rate of the reaction was slower under anerobic conditions.

Bresler et al. (97) have shown that the phosphorylated ribose nucleic acid, prepared by the previously described procedure (98), in the presence of creatine transphosphoferase, transferred its phosphate to creatine phosphate.

Spirin et al. (99) studied the DNA and RNA composition of 19 bacterial species by means of quantitative paper chromatography and ultraviolet

spectrophotometry. The DNA composition varied greatly from the extreme adenine-thymine type to the highest guanine-cytosine type. Closely related species were shown to have only small differences in the DNA composition, and definite but small differences were found even within the same genus. The RNA composition varied only slightly between species; small differences were found only between distant species. In contrast to DNA, the composition of RNA varied much less from species to species, indicating lower specificity of total RNA. Comparison of the composition of DNA and RNA indicated absence of any correlation between the nucleic acids, although it appeared probable that such a correlation exists. This correlation was expressed in a certain tendency for the RNA ratio of guanine + cytosine/adenine + uracil to increase during the transition from species with a lower ratio of guanine + cytosine/adenine + thymine in their DNA toward species with a greater value for this ratio.

Znamenskaya (100), in a study of artificial nucleoprotein formation by reserve proteins, showed that edestin and glycinin can bind, under similar experimental conditions, more DNA than RNA. The decrease in the number of basic groups of the proteins by deamination or benzoylation resulted in a reduction of their capacity to bind the nucleic acids. It was concluded that the carboxyl groups of the proteins apparently participate in the nucleoprotein formation, and, when these groups are esterified, the interaction with the nucleic acids is facilitated. The increase in the number of guanidine groups in the proteins did not alter their capacity to bind DNA, but the capacity to bind RNA was increased. Reduction of edestin with hydrogen increased its capacity to bind DNA, but the capacity to bind RNA was decreased. The RNA-protein complex of edestin, when allowed to interact with DNA, was transformed into a DNA-protein complex. The interaction of proteins with a highly polymerized DNA proceeded differently, compared to less polymerized DNA.

Spirin *et al.* (101), in studies of the composition of DNA and RNA of intestinal bacteria and their various forms, showed that saccharolytically inert forms of bacteria can be sharply classified into two groups on the basis of the composition of their DNA and RNA and other properties. One is the so-called "neutral" form (close to that described as the guanine form), and the other is the "alkali-producing" form. It was shown that the transformations of the intestinal bacteria into "neutral," or the "alkaliforming," types is accompanied by a large shift in the nucleotide composition of the DNA. Thus, the low guanine-cytosine type of DNA of the original intestinal bacteria (guanine + cystosine/adenine + thymine = 1.1) was transformed into a high guanine-cytosine type when it was transformed into the "alkali-producing" form (guanine + cytosine/adenine + thymine = 2), and into the adenine-thymine type when it was transformed into the "neutral" form (guanine + cytosine/adenine + thymine = 0.7-0.8). These changes in the DNA compositic . correlated with the changes in the heredi-

tary characteristics, namely, with the transformation of the antigenic structure, rebuilding of the enzyme complex, and alterations in the susceptibility to antibiotics. The similarity of the cultures of the same form in their biochemical properties (resistance to antibiotics and identity in antigenic structure) was accompanied by the similarity in the composition of the DNA of the cultures. In contrast to DNA, the composition of RNA was not altered during the profound changes in the biochemical and antigenic properties of the bacteria. However, the transformation into the "neutral" forms was accompanied by a definite, although not drastic, change in the RNA composition, with a tendency toward a decrease in the ratio of guanine + cytosine/adenine + uracil. According to Chargaff (102), unequivocal definition of the nucleotide sequence in a high molecular DNA is not yet possible. Procedures, however, exist which permit distinction between DNA preparations of different origin having identical base composition. Deoxyribonuclease method, i.e., that of partial degradation of DNA with the formation of apurinic acids which are more accessible to further analysis, and the analysis of differential distribution by means of acid hydrolysis of DNA, are such procedures. Biologically, it was shown that the DNA of the wild form of *Escherichia coli* rehabilitates the metabolic defect of a mutant form of this bacteria which requires lysine and, under normal conditions, is lethal (the so-called redintegration phenomenon).

Zbarsky (103) investigated the purine and pyrimidine content of DNA obtained from rat sarcoma M-1 (the growing and necrotic zones), Brown-Pierce tumors of rabbits, rat liver and spleen, the spleen of tumor-bearing rats, the liver and spleen of tumor-bearing rabbits, and calf thymus. No differences were detected in the composition of DNA from tumors and normal tissues or the growing and necrotic zones of tumors of animals of the same species. Slight, but statistically insignificant, differences were observed in the DNA composition from animals of different species. The ratio of purines to pyrimidines was found to be close to one, and the ratio of adenine plus thymine to guanine plus cytosine from the DNA of calf thymus was close to that found for the thymus of the rat, rabbit, and swine by Chargaff & Lipshitz (104).

Szymova (105) demonstrated the phosphorylation of glucose and glucosamine by the acetone powder of *Mycobacterium phlei*. The same author (106), in experiments with P^{32}-labeled KH_2PO_4, confirmed the transfer of phosphate groups from polyphosphate to glucose. It seems very probable, however, that high energy phosphate was also involved in the transfer reaction. Bukhowicz & Belozersky (107), employing P^{32}, showed that under aerobic conditions yeast synthesizes polyphosphates. Reasons were furnished for the belief that the initial synthesis of polyphosphates occurs in an acid-insoluble fraction with the eventual transition into the acid-soluble one. The inhibition of oxidative phosphorylation by dinitrophenol completely inhibited the synthesis of polyphosphates in yeast. Korotkoruchko (108) reviewed

the synthesis, content, and utilization of purines and deamination and oxidation of purines in benign and malignant tissues. Krivitskiĭ (109) reviewed the subject of nucleic acids and virus multiplication, and Luganova & Seitz (110) provided the data on the nucleic acids, phospholipides, and phosphoproteins of humar. leucocytes.

Hormones.—Yudaev & Pankov (111) described a simplified method for the determination of 17-oxycorticosteroids in the plasma of peripheral blood, based on the reaction of corticosteroids with phenylhydrazine. Yudaev & Rodina (112) reported that the synthesis of corticosteroids by adrenal slices from endogenous and exogenous precursors proceeds with greater intensity in scorbutic than in normal guinea pigs. Experiments with slices, supplemented with progesterone or deoxycorticosterone, showed that the activity of the enzymatic systems which oxidize carbons 11, 17, and 21 is not impaired during scurvy. In slices from normal and scorbutic guinea pigs, corticosterone (which usually does not occur in blood of normal pigs) was formed from progesterone. Dehydroisoandrosterone, which is a C-19 steroid, was converted in adrenal slices of normal and scorbutic pigs to C-21 corticosteroids, the unidentified C-21 steroid being the main product in control animals. An increase in the synthesis of hydrocortisone and of Δ^4-androstene-11-β-ol-3,17-dione was observed in the scorbutic adrenal slices. Ascorbic acid, added *in vitro,* did not alter the synthesis of the hormones in the adrenal slices of either group of pigs. Also, *in vitro,* the adrenal tissue of scorbutic pigs failed to respond to the supplement of adrenocorticotropic hormone (in normal tissue there was an increase in the O_2 consumption on addition of ACTH, while there was none in the scorbutic one). The conclusion was reached that ascorbic acid does not participate directly in the synthesis of steroid hormones by the adrenal tissue. Yudaev *et al.* (113) reported that the extent of incorporation of glycine-1-C^{14} into proteins of liver slices of rats which were pretreated with cortisone or deoxycortosterone is greater than in the untreated controls. Cortisone, added to liver slices in amounts of 200 to 400 μg. per 100 mg. of slice, was ineffective, and higher amounts were inhibitory to the respiration and incorporation of glycine-1-C^{14} into the proteins of the liver slices. Mednik (114), on the other hand, reported that neither ACTH nor cortisone, administered intravenously to rabbits, had any effect on the half life of the S^{35}-labeled serum proteins. Rodina (115) reported that scurvy in male guinea pigs resulted in hypertrophy of the suprarenal cortex, increasing its weight one and a half times. After 25 to 27 days on the scorbutic diet, the content of ascorbic acid in the gland decreased from 70 mg. per cent in the normal to 3.7 mg. per cent in the scorbutic animal. The level of hydrocortisone in the scorbutic pig was five times that of the normal animal. The level of 17-ketosteroids in the urine of scorbutic pigs began to increase after 20 days on the scorbutic diet to much higher levels than found in the normal animal urine. The suprarenal glands of the scorbutic pigs did not respond to ACTH,

as was judged by the blood level of the steroid hormones in the peripheral blood. Afinogenova *et al.* (116) showed that the slices of adrenals of normal guinea pigs transform dehydroepiandrosterone into corticosteroids, a transformation which has previously been shown to occur in the adrenal slices of swine (117). These workers (116) further show that the incubation of rabbit adrenal slices with dehydroepiandrosterone did not produce either corticosterone or hydrocortisone. However, other steroids of the C-21 type were formed, indicating the condensation of the C-19 steroid with a two carbon compound. Dog adrenal slices did not produce C-21 steroids from dehydroepiandrosterone, but 11-oxyandrostenedione accumulated in large amounts. This compound was also formed in the adrenals of swine, guinea pig, and rabbit, and, according to the authors, it can be considered as an intermediate product in the transformation of the C-19 steroids to the C-21 type. The adrenals of monkeys and bulls did not form either the C-21 steroids or the 11-oxyandrostenedione from dehydroepiandrosterone.

Potop (118) administered 500 μg. of thyroxin every other day for 20 days to 150 gm. male rats, and found that thyroxin stimulated anaerobic glycolysis in the brain with a parallel increase in the amylase activity and of oxidative phosphorylation. The ratio of lactic acid to pyruvic acid was increased by 59 per cent. Geller (119) reported that splenectomy in rabbits leads to a marked increase in the uptake of I^{131} by the thyroid. In patients with splenomegaly, the uptake of I^{131} by the thyroid was decreased, and splenectomy in these patients brought the uptake of I^{131} by the thyroid to normal levels. The excretion of 17-ketosteroids in the urine of splenectomized rats was decreased, the mobilization of ACTH by the hypophysis was inhibited, although the content of ACTH in the hypophysis was not altered by splenectomy. Sokoloverova (120) found that the alternated and continuous irritation of young rats by sound and light lowers their resistance to alloxan, leading to a chronic form of diabetes with lowered incidence of recovery. By similar means, a relapse of diabetes was induced in animals which recovered from alloxan diabetes. Introduction of sex hormones into the animals, prior to alloxan, also decreased their resistance to alloxan. Particularly effective was the combined neurogenic and hormonal treatment in the production of lowered resistance of rats to alloxan. Ossinskaya (121) described a differential fluorescence-analytical procedure for the determination of epinephrine, norepinephrine, and compounds with some properties of the oxidation products of epinephrinelike substances. Norepinephrine was detected in rabbit heart, spleen, skeletal muscle, brain, and liver. Epinephrine was found only in the suprarenals. Sokoloverova (122) found that immature rats are resistant to the doses of alloxan which are diabetogenic to three- to six-month-old rats and to those of over one year of age. In animals under one month of age, alloxan diabetes is of short duration, lasting only 3 to 19 days, followed by complete recovery. Histological examination of all rats confirmed the biochemical observations.

Miscellaneous.—Verkhovtseva & Surikova (123) described a quantitative procedure for the determination of true vitamin B_{12} by means of biological autochromatography. Kaleja (124) evaluated the synthesis of vitamin B_{12} by the aerobic microflora of human intestinal tract. Of the 194 types of organisms isolated, 26.9 per cent were able to synthesize the vitamin. Makarevich & Laznikova (125) reported that the propionic acid bacteria Shermanii, grown on the medium containing corn extract or yeast autolyzate, in the presence of cobalt salts, synthesize a group of B_{12} vitamins, including cyanocobalamine and the pseudovitamins. In the presence of 5,6-dimethylbenzimidazole only cyanocobalamine is formed. According to the authors, apparently all the pseudovitamins formed by the Shermanii are transformed into the cyanocobalamine by the action of 5,6-dimethylbenzimidazole even after 60 to 72 hr. following the initial inoculation of the medium, raising the concentration of cyanocobalamine from 253 μg. to 547 mg. per gm. of dry weight of the bacteria.

Manski & Zawisza (126) studied *in vitro* the cytotoxic action of various sulfonamides, in which the $-SO_2NR_1R_2-$ group was situated in the *o-*, *m-*, or *p*-position of the pyridine nucleus, on the 14-day old Crocker mouse tumor and heart tissue of a 14- to 19-day-old chick embryo, concluding that parasubstituted derivatives of pyridine sulfonamides are likely to have the most selective effect on the neoplastic tissue. Lutshnik (127) irradiated rats with x-rays (12.5 r/min.) and mice of several strains and pea shoots with γ-rays of Co^{60} (1 to 50 r/min.) and studied the effects of various yeast extracts on the LD_{50} of mice and rats and the percentage of pathological mitosis and inhibition of growth in plants. The extracts proved effective when applied after irradiation and they did not show species specificity (in contrast to other preparations from spleen and bone marrow). The more effective extracts were obtained from yeasts which were damaged by cold, drying, and irradiation, the extent of damage being such that the life of the yeast cells was depressed but not totally destroyed. The active component of the yeast extracts appeared to be RNA and its derivatives, the alleviation of the irradiation damage resulting from either a specific RNA or a depolymerized product which lost its specificity. This explanation by the author (127) is consonant with those of others (who employed spleen or bone marrow preparations) and with previously expressed views by the author (128) in regard to the involvement of RNA in the regeneration of damage induced by irradiation or by other agents (129). Seliverstova (130) reported that immediately after x-ray irradiation (800 r/min.) of *Saccharomyces cerevisiae* and *Torulopsis utilis,* a much larger amount of pantothenic acid is liberated into the medium than before the irradiation. The capacity of the cells to accumulate pantothenic acid decreased with the increase in irradiation time and dose. The capacity to synthesize the entire molecule of pantothenic acid by subsequent generations of the irradiated cells of *T. utilis* decreased, suggesting alterations by the irradiation affecting the co-

enzyme A of the cells. Trifonova (131) found that the resistance of smelt eggs and planaria to various damaging agents (high temperature, changes in pH, alcohol, and mercuric chloride), with consequent adaptation to the effect of the agents, resulted in an increase in the extent of glycolysis accompanied by a decrease in respiration. The shift in the Embden-Meyerhof pathway and in the Pasteur effect was accompanied by an increase in the rate of synthesis of nucleic acids and proteins, the latter, according to the author, accounting for the increased adaptability of the organisms to the effects of the damaging agents. According to Shtern *et al.* (132), the greatest permeability to I^{131} and P^{32} was exhibited by the liver, and the least by the brain, of white mice. After a single whole body irradiation with x-rays (800 r), an increase in the permeability during the first minute was observed in the liver, during the first 15 min. in the muscle, and during the first 45 min. in the brain. The extent of incorporation of P^{32} into high molecular weight compounds (acid-soluble extracts) decreased during the first few hours after irradiation. Bagramyan (133) reviewed the protective and curative effects of hormones in animals and man exposed to ionizing radiations, while T.rusov (134) discussed the physicochemical mechanisms of the biological effects of ionizing radiations.

Semenoff & Tregubenko (135) reported that chelating compounds, which form with cations water-soluble complexes and which are stable in the living organism, exert a pronounced effect on the behavior of the radioisotopes of Y, Ce, and Pu in mammalian organism. The most efficient complexons for the acceleration of the excretion of Y were found to be uramildiacetate and EDTA, and for Ce and Pu, hexametaphosphate. The latter markedly decreased the deposition of Ce in the skeleton. The stability constants, the rate of the chelating process, and the physicochemical state of the metal in body fluids were ascertained to be of primary importance in determining the efficiency of the complexons. Hexametaphosphate, although highly effective against Ce and Pu, was of limited value because of its toxicity, while uramildiacetate was recommended as a superior product to EDTA as a therapeutic agent in heavy metal radioisotope poisoning.

Reviews.—Stepanenko (136) reviewed the available data, including his own, pertaining to chemical structure of glycogens; Shaposhnikov (137), the topic of bacterial photosynthesis in relation to evolution of metabolism; Kursanov (138), the root system of plants as an organ of metabolism; Khvedelidze (139), the question of bioelectric potential in plants; Torchinskiĭ (140), the role of functional thiol groups of actomyosin in the mechanism of two-phase activity of muscle in the presence of ATP; Kedrovskii (141), the role of cell nucleus and cytoplasm in the molecular structural differentiation of tissues; Eskin (142), hypophysis and growth; Friedenshtein (143), the histogenic factors in bone formation; Stepanyan-Tarakanova *et al.* (144), the role of the nervous system in pathogenesis of various forms of obesity and its changes during therapy by diet; Kolotilova

(145), the oxidative pathways of carbohydrate degradation in microorganisms and animal tissues; Genes (146), modern views on the mechanism of action of insulin on metabolism; Oivin (147), the mechanism of capillary permeability; Zhukov-Verezhnikov *et al.* (148), the biological and physicochemical laws of inheritance; Yaguzhinskaya (149), the mechanism of action of insecticides; Genes (150), the mechanism of thyroxin action. Oparin (151) critically appraised the more recent experimentations and deductions on the subject of the origin of life.

A review by Zhinkin & Mikhailov (152), available in English translation (153), gives an excellent account of the developments of the work of O. B. Lepeshinskaya and her "new dialectic-materialistic cell theory," which was widely acclaimed in the U.S.S.R., was favorably assessed by several members of the Academy of Sciences of the U.S.S.R. (A. Oparin, E. Pavlovsky, A. Speransky, N. Anichkov, among several others), and was awarded a Stalin Prize, first class. Zhinkin & Mikhailov (152) summarized the data, some of which we described in a previous review (154), which explode the theory and the "facts" of Lepeshinskaya and of her supporters, thus placing the episode in the correct perspective.

An editorial (155) reviewed the progress in the various life sciences, including biochemistry, during the period of 1917-1957 and listed the names of Soviet scientists. Dunham & Stewart (156) reported that more than 200 stock tumors are maintained in various laboratories outside the U.S.S.R. To supplement this information, Pogosiyantz (157) reported that of the 43 different strains of tumors available in the U.S.S.R., 8 were obtained from abroad and all the rest of the tumors were originally obtained by the various workers in the U.S.S.R. listed in the article. Novikov (158) reviewed the work on oncology in the U.S.S.R. and found that certain important medical institutes possessing all the necessary facilities for scientific work on the vital problem of oncology do not preoccupy themselves with it. The names of the laggard institutes are cited.

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